Cloning a Fluorescent Gene

Student's Manual

Cloning a Fluorescent Gene Laboratory Manual

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genotyp

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Introduction

Today's scientists seek to expand the frontiers of human knowledge by gaining new insights into complex unanswered questions. Especially since the 1990s, the field of biology has been expanding exponentially, resulting in an incredible repertoire of very specific tools that are being used in various fields to investigate the detailed properties of living organisms.

In this rapidly expanding field of scientific inquiry, much more is expected of biology students than ever before. The foundation of basic concepts and techniques must be well-formed during secondary and early collegiate education in order for students to be able to address the more complex problems that will arise in their later training.

This laboratory course is designed to give students a first glimpse of the common technique of cloning, which is used by scientists to genetically engineer proteins and organisms, as well as to familiarize them with the incredible history of these fundamental tools. In science, the best understood systems of today become the tools of tomorrow, as every question answered allows an entirely new set of questions to be addressed. In this context, our knowledge of biology can in many ways be equated to the knowledge of the tools used in biology, because we can only answer the questions which we have the technology and knowledge to address.

The biological tools covered in *Cloning a Fluorescent Gene* have been invaluable in their applications to modern biology. For example, before this technology, people with diabetes who needed insulin injections used to get their insulin from pigs or horses. Animal insulin didn't work nearly as well as human insulin, but there was no reliable source for the human protein. Collaboration between Eli Lily and Genentech (two biotechnology corporations) generated bacteria which produced human insulin because the gene had been cloned into *E. coli* and the bacteria could now produce the human protein. The generation of large amounts of human insulin has made the lives of many people with diabetes much easier and happier.

Since the generation of recombinant human insulin, many other genes have been cloned into bacteria. These have then been used to treat human diseases or perform basic research. A total of four different Nobel Prizes in Medicine and Chemistry have been awarded to scientists for pioneering the tools discussed in this laboratory course.

Now you will get the opportunity to use these tools of biology to genetically engineer the bacteria *E. coli* to express Green Fluorescent Protein (GFP). To do this you will make use of a combination of four basic techniques.

1. PCR Amplification: You will amplify the GFP gene's DNA using **Polymerase Chain Reaction**

PCR Amplification
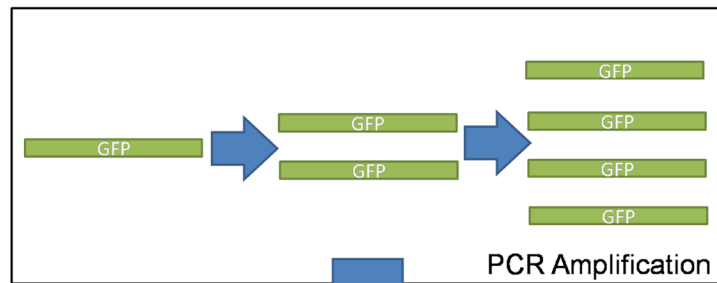
2. Plasmid Ligation: You will 'paste' GFP DNA into a linear **plasmid vector** that includes a **selection marker** and an **origin of replication**.

Plasmid Ligation

3. Bacterial Transformation: You will **transform** chemically competent *E. coli* with your ligated vector.

Bacterial Transformation
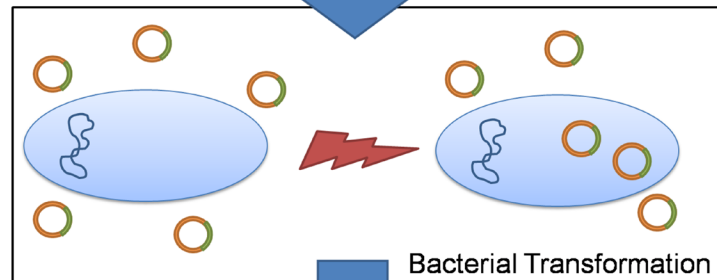
4. Antibiotic Selection: you will select bacterial **colonies** that have taken up your GFP containing vector.

Antibiotic Selection
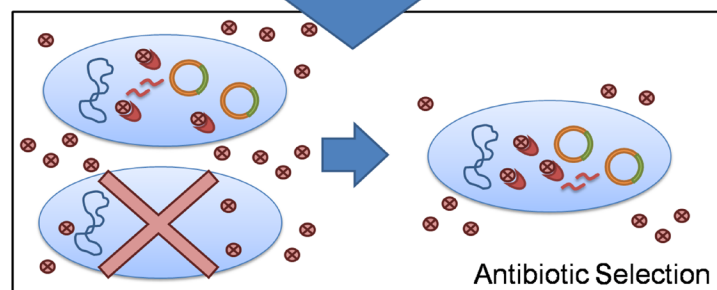
Work carefully and purposefully throughout the course, read and understand the material, ask questions when you don't understand, and enjoy yourself!

Chapter 1: Pipettes and Pipetting; Handling Samples

Introduction

Before getting started with the cloning of the fluorescent gene GFP into bacteria, you must first become acquainted with the tools and equipment that you will use during this laboratory sequence. In this laboratory session you will learn how to correctly use adjustable volume air displacement pipettes to transfer precise volumes of liquid from one vessel into another.

Background and Key Words

In the biological sciences researchers often work with difficult-to-produce enzymes and expensive reagents or tiny amounts of material such as single cells or small amounts of DNA. To perform experiments with expensive reagents or small amounts of substrate, it is useful to be able to have reaction volumes be very small, both to minimize the amounts of reagents needed for each reaction and also to avoid losing small samples. To work with very small volumes measured in intervals of $1/1000^{th}$ of a milliliter, a **microliter**, scientists use special devices called **air displacement pipettes** to lift and move tiny volumes of liquid.

A microliter, abbreviated with the Greek letter mu (µL), is $1/1000^{th}$ of a milliliter (mL) and $1/1,000,000^{th}$ of a liter (L). Keep in mind these metric system conversions when considering how much liquid you will be moving. It is helpful to remember that a milliliter is the volume of a cube with one centimeter sides. Therefore, the volume of a microliter is the volume of a cube with one millimeter sides.

Air displacement pipettes have an air-filled channel in their barrel, which can be expelled by pushing a piston into the chamber. Then, when the piston is released, it will create a partial vacuum inside of the barrel, drawing air at the open end into the pipette. By affixing a disposable tip to the end of the barrel, if the tip is placed in a liquid, then the partial vacuum can draw air into the barrel from the tip, which draws an equal volume of liquid from a reservoir into the tip
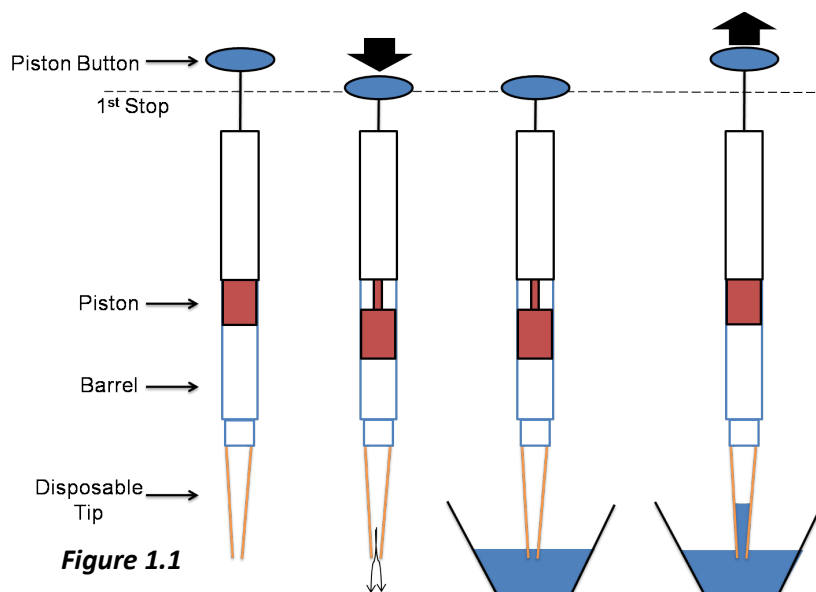
Key Words
Microliter
Air Displacement Pipette
Transfer Pipette
Microcentrifuge Tube
PCR Tube

Figure 1.1

Pipette Gear

Tens Digit
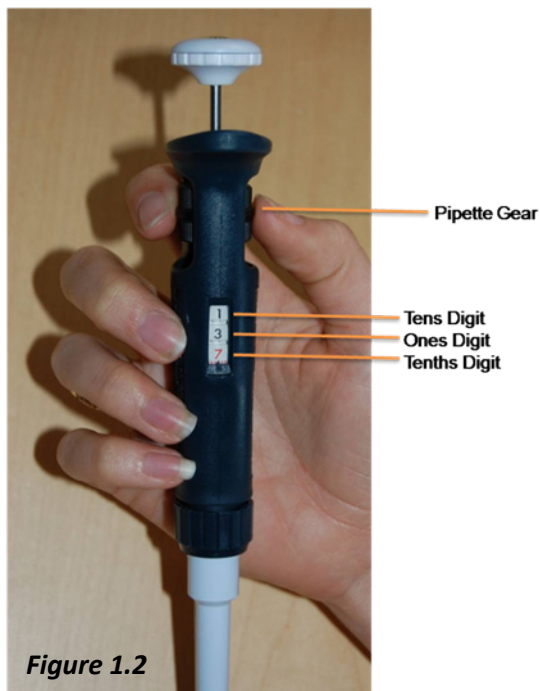Ones Digit
Tenths Digit

Figure 1.2

(Figure 1.1). Once the liquid is contained within the tip, it can be transferred to another vessel.

The amount of air expelled or drawn in by the pipette is determined by setting the distance that the piston travels down the barrel. On the side of the pipette is a gear that allows you to set the exact volume you would like to expel from the end of the pipette. By twisting the gear, it is possible to increase or decrease the total air displaced with one press of the piston (Figure 1.2).

Most air displacement pipettes are designed with a piston that has two 'stops.' The desired volume is expelled at the first stop, but by pushing the piston further purges the tip completely. (Chapter 1 video). Remember when using an air displacement pipette not to press the piston to the second stop before drawing liquid into the pipette tip, or too much liquid will be drawn in.

Also remember that the air displacement pipette should **never** be touched by the reagents that you are working with in the laboratory. The pipette must **always** be used with a tip properly affixed to its end (Chapter 1 video). Additionally, be sure to change to a fresh tip every time you switch to a new reagent! Don't contaminate the reagents that you are sharing with others!

The other liquid transfer apparatus you will be using during this laboratory course is called a **transfer pipette**, a plastic tube with a soft bulb that is squeezed to expel air, inserted into liquid, and released to draw that liquid into the tube and bulb of the pipette.

The reaction tubes you will be using to withdraw and deposit liquids come in two sizes. **Microcentrifuge tubes** can hold up to 1.5 mL of liquid (1,500µL) and are named because they are designed to fit into microcentrifuges, devices that rotate up to 14,000 times per minute to force the contents of the tube to its bottom. The other type of tube is called a **PCR (Polymerase Chain Reaction) tube**, which, depending on the size, holds 0.5 milliliters (500µL) or 0.2 milliliters (200µL) (Figure 1.3), and is perfectly sized to fit into the blocks of thermal cyclers used in PCR, which you will learn about in the next chapter.

1.5mL

0.5mL

0.2mL

Figure 1.3

Pre-Lab Questions

1. How many microliters (µL) are there in 2.0438 milliliters (mL)?

2. Should you ever use an air displacement pipette without a tip attached? [Hint: No, you never should. Touching a liquid to the end of the air displacement pipette contaminates the instrument.]

Lab 1: Pipettes and Pipetting

Purpose
In this lab you will acquaint yourself with an air displacement pipette, learning how to correctly use the device. You will also dispense all of the reagents you will be using into microcentrifuge tubes to make them easily accessible for future use. To get used to using the pipette, you will start out with a simple exercise of mixing colored acid and base solutions using an indicator called Phenol Red.

Materials
Equipment
- 2-20µL Air displacement pipette (recommended: one instrument per 2 students)
- Pipette tips
- 12 Microcentrifuge tubes for 4 students
- Plastic bag (1 bag per 4 students)
- Permanent Marker (preferably black)

Reagents
- Small beaker of water
- Phenol Red Solution (1 tube for each group of 4)
- Acid Solution (1 tube for each group of 4)
- Base Solution (1 tube for each group of 4)
- Assorted stock solutions of various reagents from *gen.otyp* on instructor's bench.

Methods
In this laboratory session you will be taking small amounts of reagents, called **aliquots**, for future experiments. The members of your lab area (usually 4 students organized into 2 pairs) will assemble all the reagents that you will need for the rest of the lab. **Every student should individually perform steps 3-5.**

1. **Choose Your Partner and Group**
    a. You and your partner will share an air displacement pipette. **Your group of 4 students will share** the reagent **aliquots** that you are going to create in today's lab.
2. **Watch the Chapter 1 video and pay careful attention to the instructions in the video.**
3. **Practice changing the microliter volumes on the pipette.**
    a. Change the volume of your pipette to 7.5 microliters (µL).
    b. Change the volume of your pipette to 12.0 microliters.
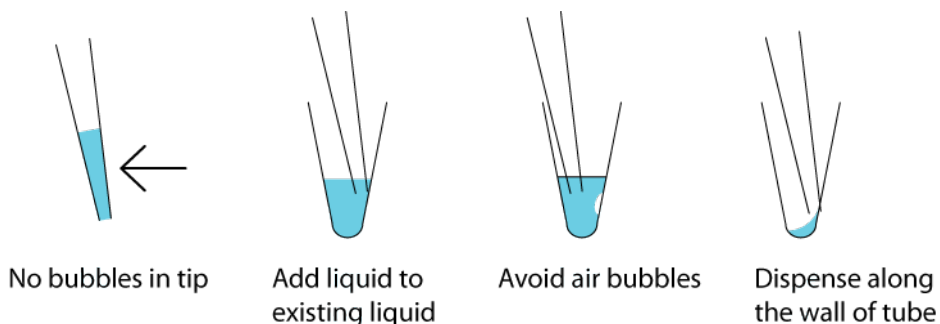4. **Practice adding liquid to the microcentrifuge tube.**

No bubbles in tip   Add liquid to existing liquid   Avoid air bubbles   Dispense along the wall of tube

***Figure 1.4. Pipetting Tips* (no pun intended)**

a. Ready an empty microcentrifuge tube to practice mixing acids and bases. Every member of the group should practice on their own microcentrifuge tube.
b. Set your pipette to 10.0 microliters.
c. Draw in 10.0 microliters of yellow Phenol Red solution into a pipette tip. Observe the level of yellow solution in the pipette tip. There should be no bubbles in your tip.
d. Expel the 10.0 microliters of Phenol Red into the bottom of the microcentrifuge tube. Observe the droplet on the bottom of the tube. It should be a yellow color. ▢
e. Eject your tip and put a new one on your pipette. You are now ready to add liquid from a new solution.
f. Add 5.0 µL of Base solution to the tube, pipetting up and down carefully to mix the liquids. It should now be a fuchsia color. ▢
g. Change tips and add 10.0 µL of Acid solution to the tube. It should return to its original yellow color. Make sure to mix the solution until the entire solution is yellow. ▢
h. Change tips and add 15.0 µL of Base solution to the tube, it should return to its fuchsia color. ▢

5. **Practice removing liquid from the microcentrifuge tube.**
   a. Set your pipette to 7.5 microliters.
   b. Touch the tip to the fuchsia liquid and draw in 7.5 microliters of liquid.
   c. Make sure you have not drawn in any air bubbles in the pipette tip.
   d. Eject the pipette tip. You are now trained to use an Air Displacement Pipette.

6. **Assemble the reagents that you will use for your group of 4 students**
   a. Number, initial, and label eight microcentrifuge tubes using a permanent marker according to the following table, and add the stated volumes from the stock solutions on the instructor's bench to each tube. Be sure to carefully add the volumes to the bottom of the labeled microcentrifuge tube, because you will need almost every microliter for your group's experiments. To add more than 20.0µL to a tube, simply set the pipette to 20.0µL and perform multiple transfers. **Change tips for every different reagent!**

Label	Volume (µL)
Template	25.0
5x PCR Buf.	25.0
DNTPs	30.0
GFP For.	18.0
GFP Rev.	18.0
Vector	25.0
5x Lig Buf.	20.0
Water	40.0

   b. Divide the tubes among the different members of your group. One member is responsible for getting an aliquot of one reagent for the entire group. At the end of this step, your group of four should have 8 tubes total, each for a different reagent. Your group will share these reagents in Chapter 2 and Chapter 3.

7. **Store your reagents**
   a. Close the lids of each tube tightly
   b. Gently put all of the tubes into a sealable plastic bag (such as a Ziploc® bag)
   c. Label the bag with initials of group members, and give it to your instructor to be placed in the freezer until needed.

Clean-up

Make sure that each tube that you have dispensed your reagents in is clearly labeled with your group's initials and the name of the reagent. Place all of the tubes gently in the small plastic bag and label the

bag with your initials. The bag should be placed in the freezer until the next laboratory session, when some of the reagents will be used.

Conclusions

Now that you have some experience using air displacement pipettes and microcentrifuge tubes, you are capable of performing all the laboratory techniques required in the next two lab sessions. Though you may not understand the purposes of each of the reagents that you have parceled out, they will be revealed to you soon. Starting in the next laboratory session you will use your new skills to perform a polymerase chain reaction to amplify the green fluorescent protein gene.

Chapter 2: Polymerase Chain Reaction

Introduction

Because molecular biology applications are not 100% efficient, the ability to turn one piece of DNA into millions of pieces of identical DNA is an important tool for researchers. DNA sequencing, cloning, and genotyping are all research techniques demanding a huge supply of identical pieces of DNA.

PCR, or Polymerase Chain Reaction, is currently one of the most common techniques used by researchers working with DNA. This procedure allows researchers to amplify a single piece of DNA using a thermostable polymerase, which was isolated from bacteria living at very high temperatures.

In this session you will be amplifying the DNA of Green Fluorescent Protein (GFP) from a piece of DNA that already contains the GFP gene. You will then, in a later lab, be able to use this amplified DNA to put the GFP gene into an expression vector, so that the protein can be expressed in bacteria.

Background and Key Words

PCR, or **P**olymerase **C**hain **R**eaction, is used to repeatedly replicate a piece of DNA in order to increase the number of copies of a DNA sequence, a process known as **amplification**. PCR allows us to generate millions of copies of a DNA sequence in between two **Primers.** The primers are small single stranded DNA (ssDNA) fragments of the 5' ends of the PCR product that will be amplified (see Figure 2.3). The new DNA in all organisms is made by a specialized enzyme called **DNA Polymerase**. In PCR, the new DNA is generated by the DNA Polymerase **Taq**, which was isolated from the Archaebacteria *Thermus aquaticus*, an organism living at 70˚ Celsius (158˚ Fahrenheit) in the "Great Fountain" of Yellowstone National Park. During PCR, the reaction is heated to temperatures as high as 95˚ Celsius (203˚ Fahrenheit) to pull apart double stranded DNA (dsDNA). Taq Polymerase is unique because it remains stable at this high temperature; most enzymes would unfold and be rendered useless under such extreme conditions.

To better understand how DNA is synthesized, it is important to know how DNA is structured. DNA has 3 main parts, a nitrogenous base, a sugar, and a phosphate group. The backbone of DNA, which consists of the sugar and phosphate group, is identical among all four DNA nucleotides. Thymine (T), Adenine (A), Guanine (G), and Cytosine (C) differ in the structure of their
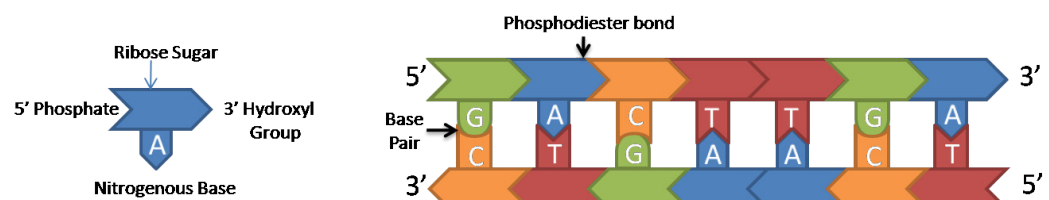
Key Words
PCR
Amplification
DNA Polymerase
Taq
DNA Directionality
Primer
5x PCR Buffer
DNTPs
Denaturataion
Annealing
Elongation/Extension

Figure 2.1

nitrogenous bases. The double stranded structure of DNA is created by the bonding of complimentary base pairs, Adenine with Thymine and Cytosine with Guanine. The phosphate group of the DNA backbone is attached to the 5th carbon atom in the sugar molecule, and there is a hydroxyl (–OH) group on the 3rd carbon in the sugar (Figure 2.1). You may think of these two groups as the 'front' and 'back' of the nucleotide. When two nucleotides connect in a line, a special bond, called a phosphodiester bond, forms between the phosphate on the 5th carbon atom, and the hydroxyl group on the 3rd carbon atom. DNA is always replicated starting with the 5' end (indicating the 5th carbon) adding onto the 3' end (the 3rd carbon). Consequently, all genes are found in the 5' to 3' direction. This concept of DNA being replicated and transcribed starting from the 5' phosphate end and adding to the 3' hydroxyl end is known as **DNA Directionality**.

The DNA used to start PCR, called the template, can come from any source that contains DNA. The DNA sequence of the template has to be at least partially known so that primers can be designed at each end of the sequence that will be amplified. In addition to the primers, a PCR reaction also requires a **PCR Buffer** to stabilize the Taq Polymerase enzyme. Many proteins use ions from salts present in their natural environment to perform their functions, so reactions

Step 1: Denaturation
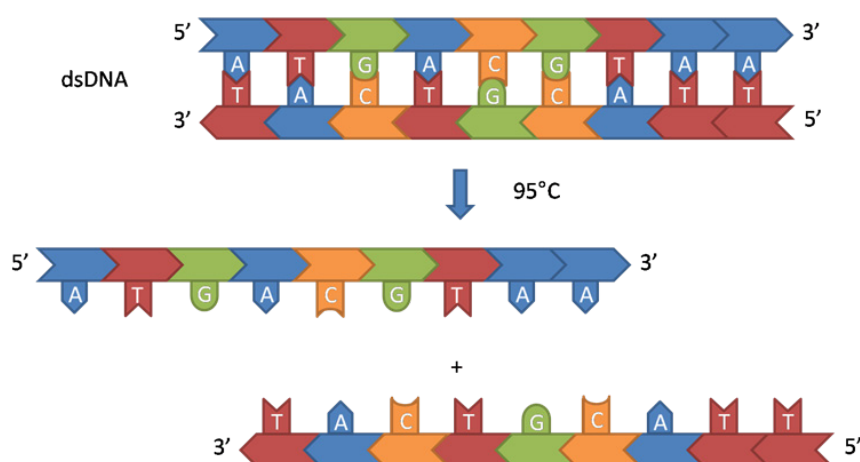
involving proteins require a buffer to supply these salts in the reaction tube. The buffer provided is called a 5x Buffer because it contains 5 times the required concentration of salts. Therefore this solution must be diluted in order for the final reaction mix to have the appropriate salt concentration.

Finally, in order to generate new DNA, a PCR reaction needs to contain **DNTPs, D**eoxyribo **N**ucleotide **T**ri**P**hosphate**s**, the building blocks of DNA. These DNTPs are DNA nucleotides with 3 phosphate groups that can be linked together to make new DNA. All of these reagents together will be cycled between various temperatures to amplify the DNA between the two primer sequences.

DNA polymerases cannot simply replicate a piece of dsDNA into two identical strands. In order to amplify DNA in a test tube we need to unzip the dsDNA into two pieces of ssDNA. This step of PCR is called **Denaturation** (Figure 2.2). In the cells of our body, this task is performed by a special enzyme called Helicase. Because we do not have access to Helicase in the test tube, we instead use high temperatures to pull apart, or *denature*, dsDNA. dsDNA becomes two strands of ssDNA at around 94˚ Celsius.

The next stage of PCR is to attach the primers to the ssDNA. This step of PCR is called **Annealing** (Figure 2.3). To do this, we simply lower the temperature of the reaction to a level where complimentary bases can bind together again, or anneal. Since there are many more copies of the primers in a PCR mixture than copies of the Template ssDNA, it is much more likely that the ssDNA template strand will bind a primer rather than a complimentary ssDNA strand as the mixture is cooled.

Now that our Primer is bound to ssDNA, we need to activate Taq Polymerase, which initializes the synthesis of new DNA. The temperature of the mixture is raised to 72˚C, where Taq Polymerase is most active, and free-floating DNTPs are recruited to make new DNA. Also remember, DNA is *always* made in the 5' to 3' direction, so the polymerase extends the 3' end of the Primer to the end of the ssDNA template. This step of PCR is called **Elongation** or **Extension** (Figure 2.4).

At the end of the Elongation step, the reaction mixture now contains twice as much dsDNA as it started with, but it's still not enough! To get much more DNA we repeat the Denaturation, Annealing, and Elongation steps up to 40 more times. In each new cycle the DNA strands made from the previous cycle are used as templates for the new cycle, effectively doubling the amount of DNA with each cycle. If the amount of DNA is doubled each cycle, you get a whole lot of DNA from just 1 strand of template (you'll be calculating exactly how much in the pre-lab questions).

Step 2: Annealing
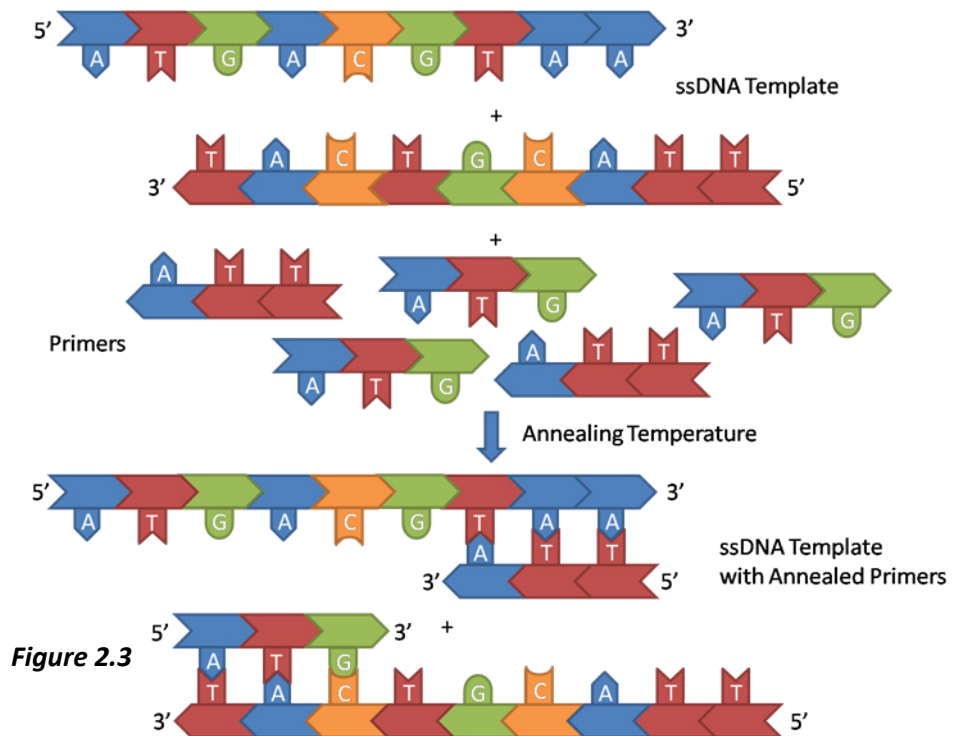
Figure 2.3

Step 3: Elongation
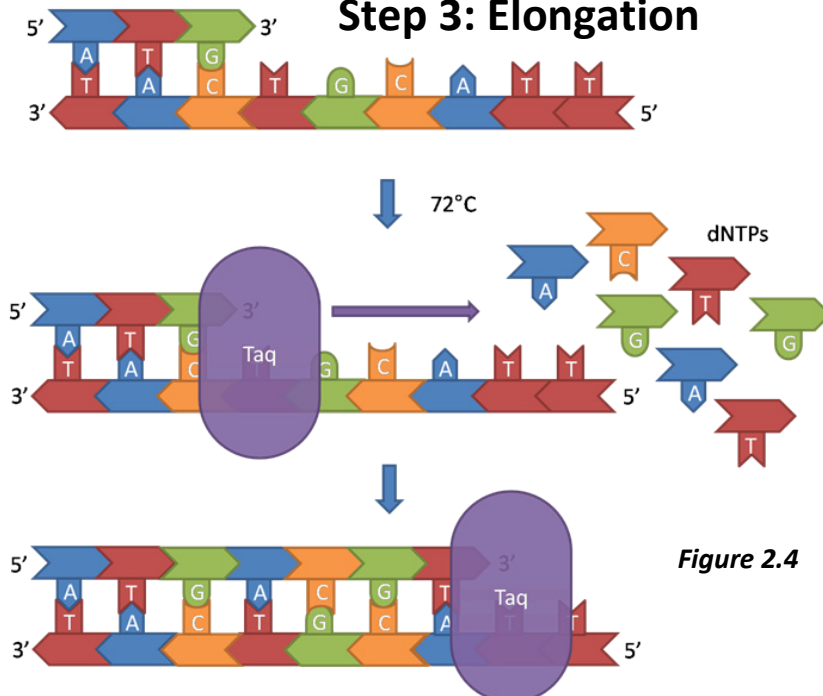
Figure 2.4

Now that you understand how PCR works, it is important to understand how only the DNA between the two primers is amplified on the exponential scale described above, since most templates used in PCR will be longer than the target sequence. This process is complicated, so it may be helpful to follow along in Figure 2.5 on the next page. The primer binds in the middle of an ssDNA strand and makes new DNA by extending from the 3' end of the primer. This replicated area is said to be "downstream" of the primer, while the area on the 5' end of the primer, which does not get replicated, is said
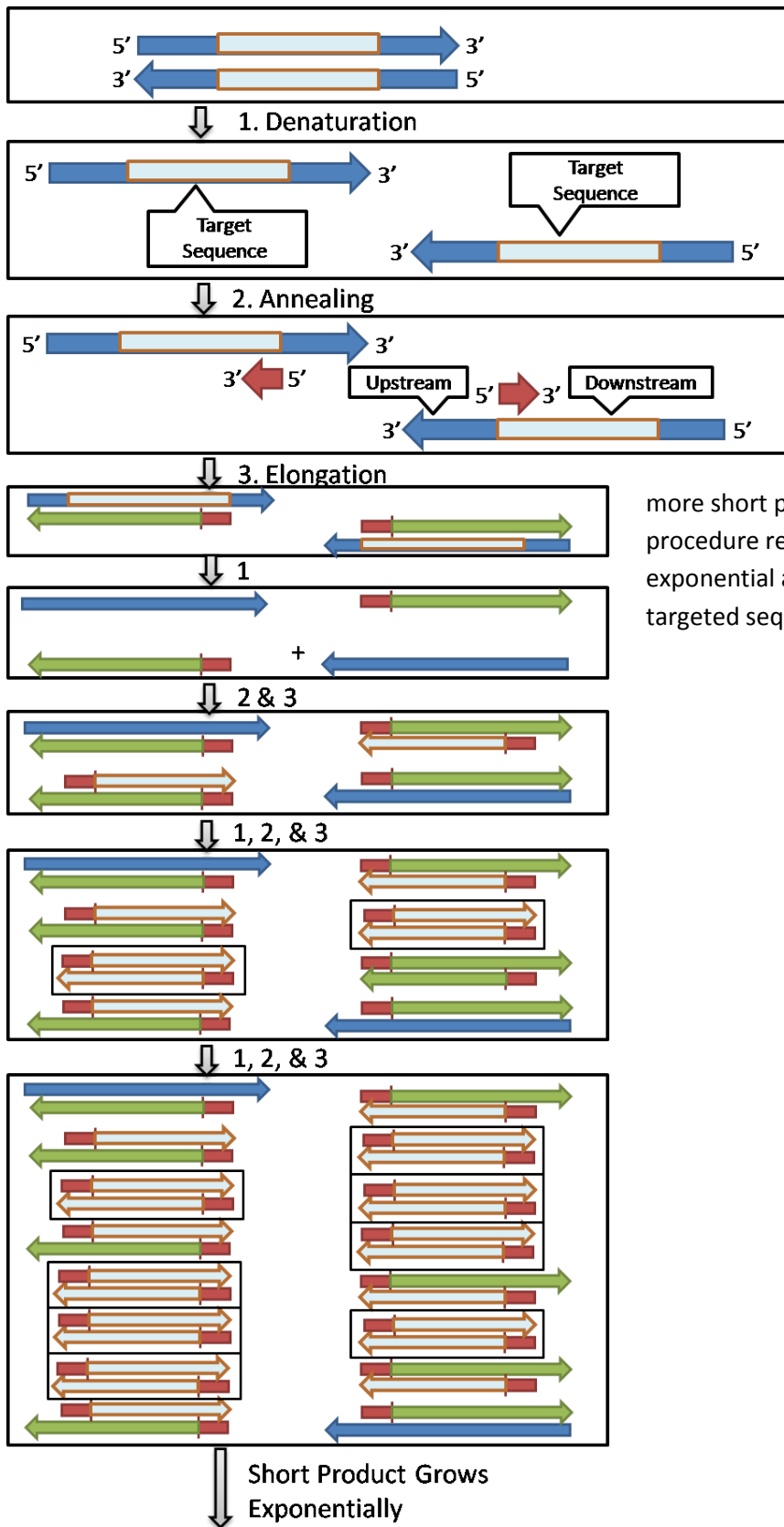
to be "upstream" of the primer. The "downstream" area of each primer is different, but overlapping. The sequence downstream of both primer binding sites will be the Target Sequence; only this piece of DNA will be amplified by PCR. Long pieces, with some upstream DNA, will give rise to short pieces, lacking that upstream DNA. Meanwhile, short pieces will give rise to more short pieces. The net result of this procedure repeated over and over is the exponential amplification of only the short, targeted sequence.

Figure 2.5

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Pre-lab Questions

1. You find out that the sequence of the GFP gene is
5'_ATGGCTAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTA
ATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCTACATACGGAAAGCTTACCCTTAAATTTA
TTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTT
CCCGTTATCCGGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAC
GCACTATATCTTTCAAAGATGACGGGAACTACAAGACGCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTG
TTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTCGGACACAAACTCGAGTACA
ACTATAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTC
GCCACAACATTGAAGATGGATCCGTTCAACTAGCAGACCATTATCAACAAATACTCCAATTGGCGATGGCC
CTGTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGCGTGA
CCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATGAGCTCTACAAAT_3'
    Design Forward and Reverse primers each with a length of 15 nucleotides that will amplify the GFP gene. Remember, T pairs with A and C pairs with G. It may help to draw a picture of the base pairs (not the whole gene!)

2. Use a scientific calculator to calculate the total number of DNA strands in the reaction mixture at the end of 35 cycles of PCR amplification starting with one strand.

3. When researchers measure how much DNA is made in a PCR reaction, they find that after a certain number of cycles the amount of new DNA created per cycle slows down and then disappears all together; this phenomenon is called *plateauing*. Think of two possible reasons that plateauing could occur.

Lab 2: Polymerase Chain Reaction

Purpose
We will use Polymerase Chain Reaction to amplify the GFP gene. Using a DNA Polymerase called "Taq" and two primers that complement the two ends of the GFP DNA, we will create a huge number of linear copies of GFP DNA. In the next lab this DNA will be inserted into an antibiotic resistant vector.

Materials
Equipment*:*
- 2-20μL Air displacement pipette (1 per 2 students)
- Pipette Tips
- 0.2mL (PCR) Tube
- Thermal Cycler (1 per class)

Reagents*:*
- Reagents from your group's bag
    - 5x PCR Buffer
    - DNTPs
    - GFP Forward Primer
    - GFP Reverse Primer
    - DNA Template
- Taq Polymerase

Methods
You will be mixing together the appropriate reagents in given amounts, just as you practiced in the first laboratory session. Use the table on the right below to reference how much of each reagent, adding each reagent in from top to bottom in the order listed. It is important to remember good pipetting skills to ensure that there is enough of each reagent for everyone in your group.

Each student should perform their own PCR reaction!

1. **Label** your PCR tube on the cap and the side with your initials using a dark permanent marker.
2. **Add the PCR reagents to the tube**. Wait for all reagents to thaw completely before pipetting.
    a. Add 4.0μL of 5x PCR Buffer to the tube.
    b. Add 5.0μL of DNTPs
    c. Add 3.0μL of GFP Forward Primer
    d. Add 3.0μL of GFP Reverse Primer
    e. Add 4.0μL of DNA Template
    f. Add 1.0μL of Taq Polymerase
    g. Mix Tube gently.

Reagent	Amount
5x PCR Buffer	4.0μL
DNTPs	5.0μL
GFP For.	3.0μL
GFP Rev.	3.0μL
DNA Template	4.0μL
Taq Polymerase	1.0μL
Total	**20.0μL**

3. Close the tube and bring it to the Thermal Cycler. Place the tube in an open tube slot. Make sure your tube is properly labeled. Once all the students in the class are finished putting

together their reactions. The instructor should start the PCR machine on the "GFP_PCR" program (the details of this program are found in the CAFG Instructor's Manual).

Clean-up

PCR Tubes may remain in the Thermal Cycler overnight, but for longer periods, they should be stored in the refrigerator. Unused reagents may be thrown out unless your instructor is collecting them. Double check to make sure that your PCR Tube is labeled with your initials so that it does not become mixed up with other tubes.

Conclusions

By the end of the thermal cycling your PCR Tube should contain a large number of copies of short sequences of DNA that encode for only the GFP gene. During the next lab period you will be inserting this short DNA sequence into a larger piece of circular DNA that can then be put into bacteria for expression.

Chapter 3: Ligation

Introduction

The genetic code of an organism must remain intact in order for new proteins to be made or for cells to divide. Damage from agents like UV light that break apart DNA can be repaired by special proteins that glue broken DNA back together.

In the laboratory we make use of these proteins, called DNA ligases, to purposely insert a new piece of DNA into an existing sequence.

In the last lab you amplified the DNA sequence of the GFP gene using PCR. In this part of the lab you will be inserting your GFP PCR product into a circular piece of DNA. This pasting of DNA is called **Ligation**. With this circular DNA containing GFP, you will be able to make bacteria produce the GFP protein.

Background and Key Words

Higher eukaryotes are unique in the living world in that they have linear chromosomes. Each of a human's 23 pairs of chromosomes is one incredibly long piece of linear DNA (50 million to 250 million base pairs long!). Bacteria, however, do not have the complex machinery needed to maintain a linear chromosome, so they use smaller, circular chromosomes.

Bacteria need their chromosomes to remain circular in order to survive, but there are a number of factors which break DNA such as chemicals, UV light, and some proteins (called nucleases). Breaks in DNA caused by these agents can interrupt genes and prevent the replication of the bacterial chromosome. In order to repair their broken DNA, bacteria have evolved to produce a protein called DNA ligase, which reattaches two broken ends of DNA into one continuous strand.

Researchers make use of the special properties of this protein to glue together pieces of DNA which originally didn't go together, generating an entirely new sequence. This process is called **Ligation** (Figure 3.1), and changing the DNA code of organisms using ligation is the core technology in genetic engineering.
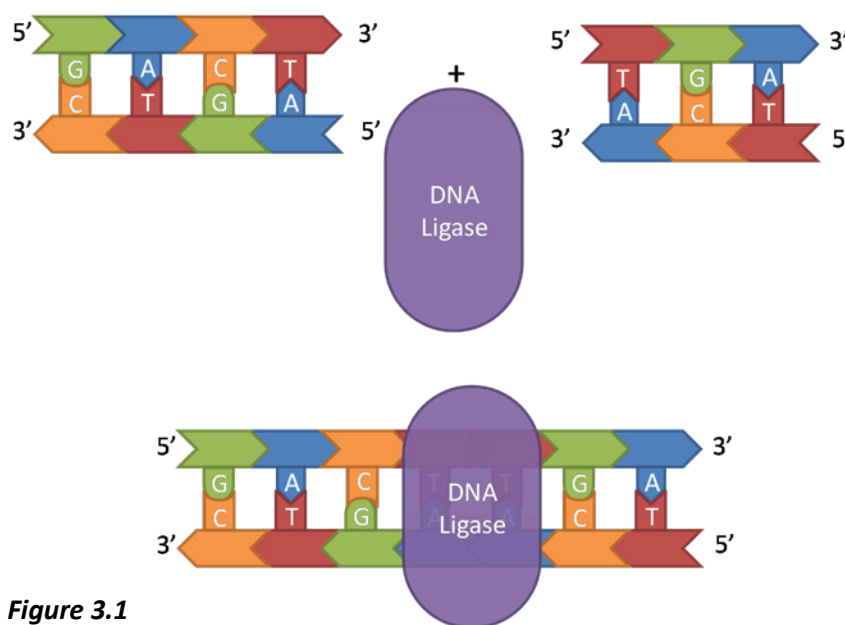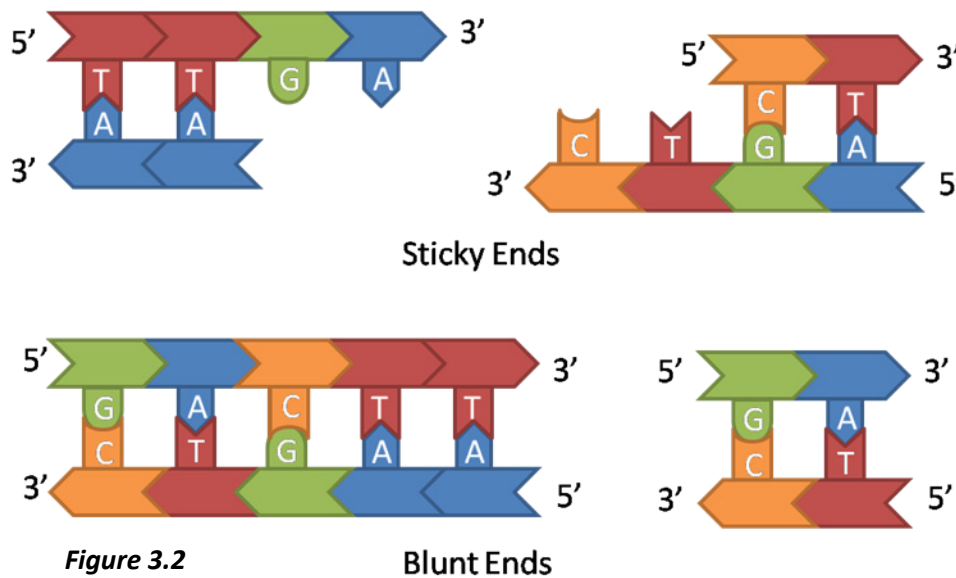
Key Words
Ligation
Plasmid
Vector
Insert
Blunt End
Sticky End
Restriction
Endonuclease

Figure 3.1

Sticky Ends

Figure 3.2   Blunt Ends

In the laboratory, DNA is most often cut and pasted into a small circular piece of DNA called a **plasmid**. Plasmids are similar to normal bacterial chromosomes except that they are a) smaller, b) contain only specific genes selected by the researcher, and c) are present in multiple copies within a cell (sometimes hundreds of plasmids per bacterium). A plasmid without a DNA insertion is called a **vector**; a vector is the vehicle used to deliver the insert sequence into the host, and then to replicate and express the sequence. In addition to the vector, the piece of DNA that will be newly pasted into the plasmid is called the **insert**. . We'll talk more about how replication and expression works inside a bacterium in the next two chapters.

Now, let's discuss the actual mechanics of attaching two pieces of DNA together using a DNA ligase. Firstly, it is important to note that DNA ligase always creates *two* phosphodiester bonds, joining both sides of the end of a dsDNA strand. The ends of linear dsDNA can come in two forms, "blunt" and "sticky." **Blunt Ends** simply end the strand as dsDNA, with no overhanging nucleotides. **Sticky Ends**, however, have one or more overhanging bases at the end of the fragment (Figure 3.2).

In DNA manipulations, scientists use sticky ends and blunt ends for different purposes. A blunt end can be attached to any other blunt end, regardless of what sequence is formed after the connection is made. Sticky ends can only be attached to other sticky ends that have *complimentary* single-stranded overhangs (Figure 3.3). This drastically increases the specificity of the ligation reaction because the ssDNA overhangs must form complimentary base pairings before the ligase can create phosphodiester bonds to paste the two pieces of dsDNA together.
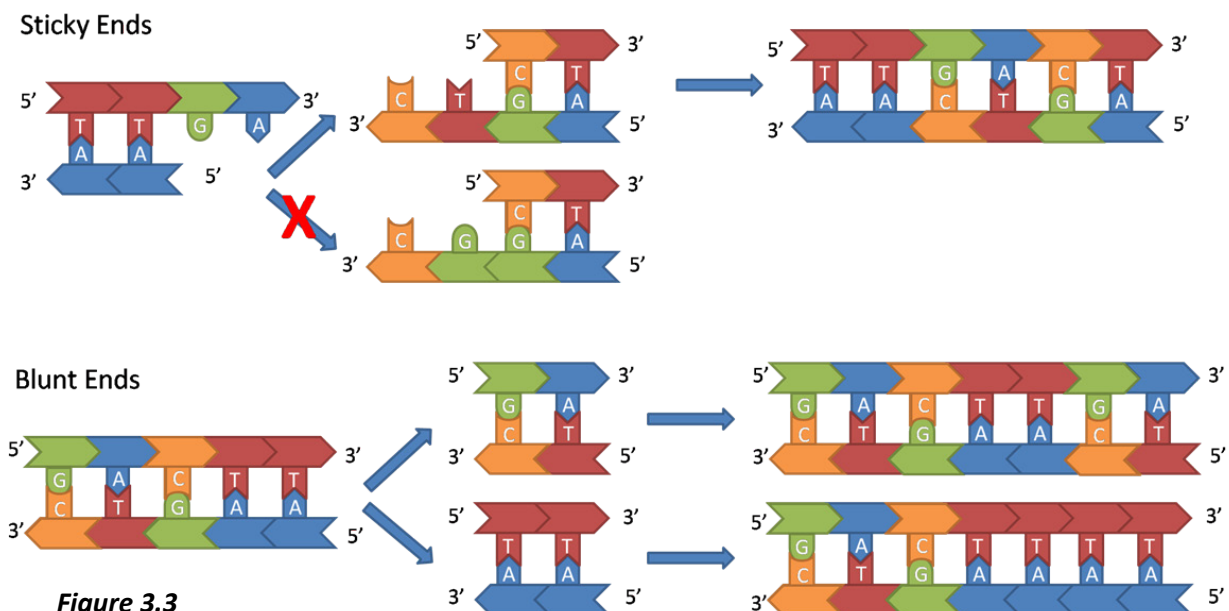

Figure 3.3

PCR Product

+

Linear Vector

DNA Ligase

Circular, Ligated Plasmid

It seems logical to assume that the DNA fragment that has been amplified by the PCR reaction is blunt-ended, but that is actually *not* the case. Taq Polymerase has a rather unusual and useful property of being able to add an additional adenosine (A) on the 3' end of DNA strands it synthesizes, resulting in a single-stranded adenosine overhang on most of the DNA fragments generated. Because of this overhanging A, the PCR product cannot bind to itself or another PCR product during a ligation reaction (because A doesn't form a base pair with A). In

Figure 3.4

order to get the PCR products to attach to another piece of DNA, that DNA must have a 3' overhanging thymine (T). The opened vector that you will be using has exactly this, a 3' overhanging T on each end, which will easily attach to the 3' overhanging A's of the PCR product. Utilizing this property of Taq Polymerase for ligation is a type of sticky ends cloning (Figure 3.4) because it connects overhanging thymine and adenosine bases to increase the specificity of the ligation reaction.

This type of cloning is a convenient method of inserting a PCR product into a plasmid vector. However, not all ligations are performed using a linear construct with overhanging T ends. Usually, researches start with a circular plasmid that has no insert, and must open the circular vector into a linear piece of DNA using a special type of protein that cuts DNA called, a **Restriction Enzyme**. These proteins recognize specific base pair sequences of DNA and cut dsDNA at those points. These enzymes recognize palendromic sequences, which read the same forwards as backwards. EcoRI, which recognizes the

Figure 3.5

sequence GAATTC (Figure 3.5), cleaves the sequence after the second "T", generating two strands with an overhanging AATT ssDNA sequence. A circular plasmid can be cut into a linear strand using EcoRI, allowing any new piece of DNA with an AATT overhang to be ligated into the plasmid, reforming a circle with a new piece in the middle. Lots of restriction enzymes exist, which recognize and cut
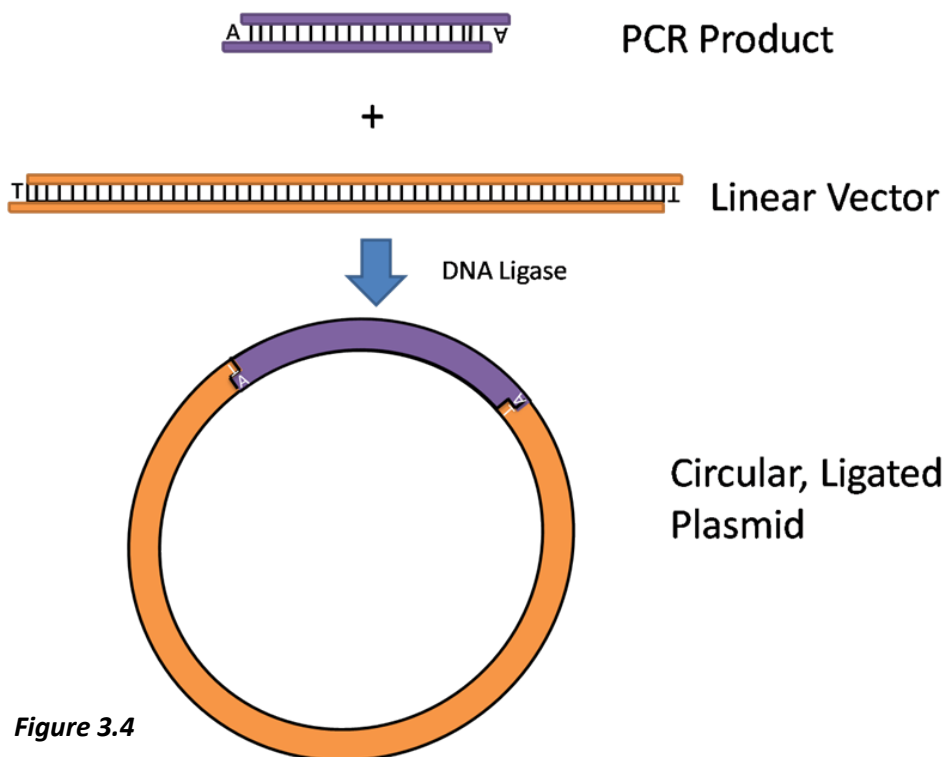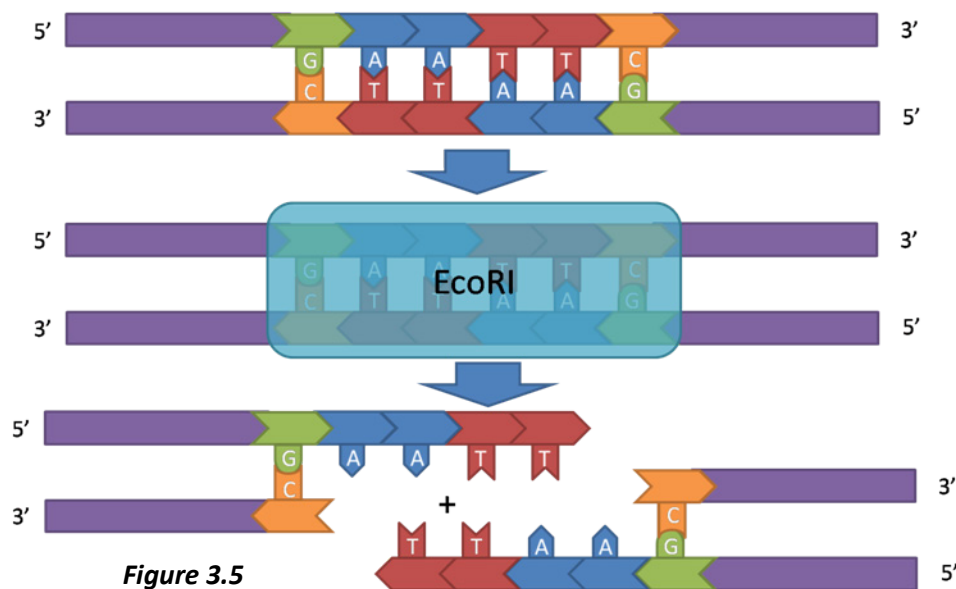
DNA differently. Would it be helpful edit figure 3.4 so that it shows the PCR product + a circular vector, then circular vector + restriction enzyme = linear vector, then PCR product + linear vector + ligase = plasmid? I think it would show the more complete process that you talk about in the page.

Pre-Lab Questions

1. Using PCR, you amplify the short sequence 5'—GATGGCATACGGAC—3' using Taq polymerase. Draw a base-paired diagram of what both strands of this PCR product will look like after Taq adds the adenosine base.

2. Now that you know about DNA directionality and sticky ends cloning, you can begin to understand DNA *orientation*. Draw two simple diagrams of the plasmid labeling the 5' and 3' strands of DNA using Figure 3.4 as a model: in each diagram show how the PCR product can be inserted in a different orientation.

3. EcoRI cuts DNA at the site 5'-G|AATTC-3' as pictured in Figure 5 above. You have two short DNA sequences with EcoRI cut sites in them:
   5'—GATCATATGAAAC<u>GAATTC</u>GCAT—3' and 5'—AC<u>GAATTC</u>ACAATCTGCCCTTTC—3'
   Draw a base-paired diagram of what each DNA strand will look like after being cut with EcoRI. Then, draw another base paired diagram of the DNA strand made from ligating the two long pieces of DNA from the EcoRI cuts you have just described.

Lab 3: Ligation

Purpose
Today you will ligate your amplified DNA from the previous lab into a linearized vector that you placed into an aliquot on the first day. This procedure should generate circular plasmids containing GFP DNA.

Materials
Equipment*:*
- 2-20µL Air displacement pipette
- Pipette Tips
- 0.2mL (PCR) Tube

Reagents*:*
- Amplified GFP PCR product (from last lab)
- Reagents from your group's bag
    - Linearized Vector
    - 5x Ligase Buffer
- DNA Ligase Enzyme

Methods

Reagent	Quantity (µL)
PCR Reaction	4.0
Water	6.0
Linearized Vector	4.0
5x Ligase Buffer	4.0
DNA Ligase	2.0
Total	**20.0**

1. **Label** your Microcentrifuge tube with your initials using a permanent marker.
2. Take out the 5x Ligase Buffer to **thaw**
3. **Add the Ligation Reagents to the tube**
    a. Use a new pipette tip for each different reagent you use, discarding the old one in the trash.
    b. Add 4.0µL of your PCR reaction to the bottom of the new 0.2mL tube
    c. Add 6.0µL of Water to the tube
    d. Add 4.0µL of Linearized Vector to your mix
    e. Add 4.0µL of 5x Ligase buffer
    f. Go to your instructor's lab table and add 2.0µL of DNA Ligase, which should be sitting on ice.
    g. Shake the tube gently so that all the components are mixed well
4. **Let the reaction sit overnight** at room temperature
5. **Watch** the Chapter 4 video to get an idea of the techniques you will be using next lab.
6. The next morning, you or your instructor will put your reaction into the Thermal Cycler **and run the "Lig End" program**, which heats up your sample to denature your enzyme, ending the reaction and improving the efficiency of transformation.
7. Place your tube back in the plastic bag for your group after running the "Lig End" program. **Store the ligation reaction** in the freezer until next lab period.

Clean-up
After the reaction has been allowed to incubate overnight, be sure to put your ligation reaction in your group's bag and place the bag back in the freezer. Extra 5x Ligase buffer, Linearized Vector, and PCR product can either be stored for another experiment or thrown away.

Conclusions

Some of the pieces of linear DNA that you have mixed together in your reaction tube should have been joined together by DNA ligase into a circular plasmid. These circular pieces will remain mixed in with the un-ligated fragments until next lab session, when you will be putting the DNA into bacteria, and then only allowing the bacteria which have taken up the circular plasmid will grow.

Chapter 4: Transformation

Introduction

Normal cells are very resistant to new DNA entering their cytoplasm. If cells simply took in any DNA around them, they would quickly become infected by viral genes that work to take over the cell's machinery for their own purposes. To defend against the entry of viral genes, cells have multiple mechanisms to stop DNA from getting into the cell. In bacteria the primary block to DNA entry is the cell membrane.

To get a piece of DNA into bacteria, we need to "trick" the cells into taking up the DNA around them. In the laboratory we can do this by stressing cells, putting them into a "panic mode". Bacterial cells in panic mode will actively take up DNA around them in an effort to save themselves from death. When we use this process to insert new genetic material into bacteria, it is called transformation.

After getting DNA into the cells, we can then set up an experiment that will allow only the cells which have taken in a complete plasmid to grow.

Disease-Causing Bacteria

Use Heat to Kill Bacteria

Mix Together

Dead Bacteria

Normal Bacteria

"Transformed," Disease-Causing Bacteria   *Figure 4.1*

Key Words
Transformation
Competence
Calcium Chloride
Heat Shock
Origin of Replication
Antibiotic
Recover
Agar Plate
Spreader
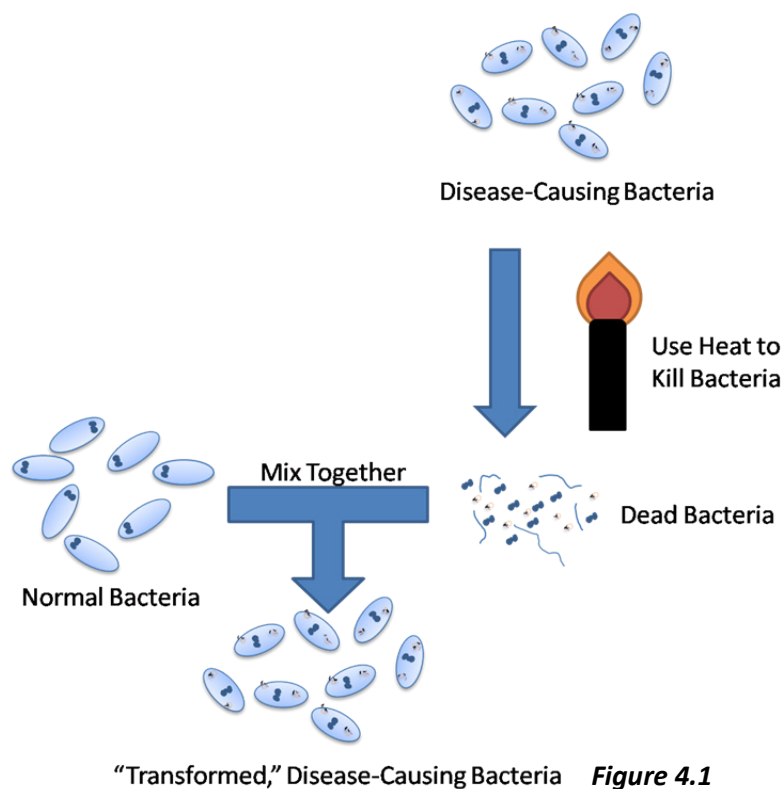
Background and Key Words

In the context of this experiment, transformation of bacteria refers to the insertion of our genetically engineered plasmid into cells. However, the idea of transformation significantly predates genetic engineering. The term was first used to describe how a non-harmful strain of *Streptococcus pneumoniae* bacteria could be "transformed" into disease-causing bacteria after being exposed to the remains of dead pneumonia-causing *Streptococcus pneumoniae* (Figure 4.1). Decades later, scientists discovered that these non-harmful bacteria were taking

Figure 4.2

up genes from the dead cells' DNA that could cause disease. This discovery led to the use of transformation in genetic engineering.

Genetic **transformation** is the process used to alter an organism's phenotype by introducing new genetic material.

The first important thing to understand about transformation is how researchers trick bacteria into accepting new DNA. After that we will move on to discuss how that DNA will behave once it is inside the cell.

Most of the time it is a very bad idea for bacteria to just take up DNA around them, because the bacteria could be transformed by genes that do not help it at all (like viral genes). Therefore, in order to get the cells to take up the engineered plasmid, they must be stressed enough to take up DNA surrounding it. When presented with a dire situation, bacteria will attempt to take in genetic material around it in order to survive the ordeal. The ability of cells to take up DNA is called **competence**, and competence can be created by two methods. Electrocompetent cells are those that will take up DNA after being shocked with a large amount of electricity. Chemically competent cells are incubated on ice in a salt solution of **Calcium Chloride** ($CaCl_2$) then quickly heated to 42˚ Celsius to shock them into taking up surrounding DNA (Figure 4.2). In today's lab we will be transforming chemically competent cells using this method, called **heat shock**.

It is now finally time to talk about how a plasmid actually functions within a bacterial cell, and what purpose different parts of the vector have. As you know, plasmids for cloning are put together by scientists in the laboratory, so every piece of a plasmid is placed into the vector for a specific purpose. In order for a plasmid to be useful for cloning, it must have at least two parts: a selection marker and an **origin of replication** (oriC) (Figure 4.3). The selection marker we will use is a gene that encodes a protein that gives bacteria resistance to the antibiotic ampicillin, and we will discuss more about selection in the next chapter. The origin of replication is important for the generation of new copies of the plasmid within the bacterial cell.

Recall that the chromosomes of prokaryotes like *E. coli* bacteria are circular pieces of DNA, just like plasmids. Because we know that *E. coli* are capable of replicating their own DNA, it makes sense to use the same signal that starts the replication of the bacterial chromosome to replicate our plasmid. This signal is a series of nucleotide repeats in the DNA that help bind proteins responsible for replicating DNA. In *E. coli* this sequence is given the name *oriC*. Once the plasmid is inside the cell, DNA replication proteins will bind to the *oriC* of the plasmid and begin copying the plasmid. At the same time these *oriC* binding proteins start replication the DNA of the bacterial chromosome (Figure 4.4). Thus, by utilizing the replication machinery of the bacteria, we can copy our plasmid inside live cells.

Transformation and plating of bacteria requires four steps. In order to prepare cells for heat shock, we must first mix them with the plasmid DNA
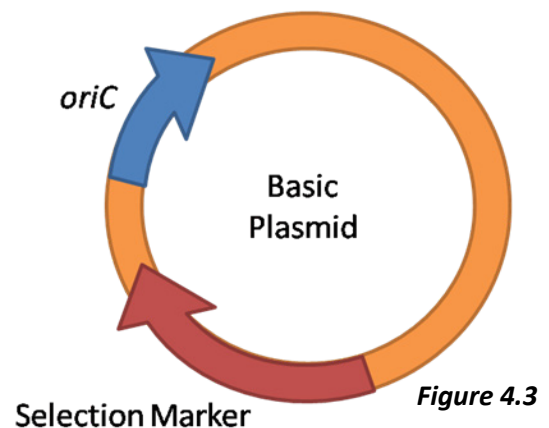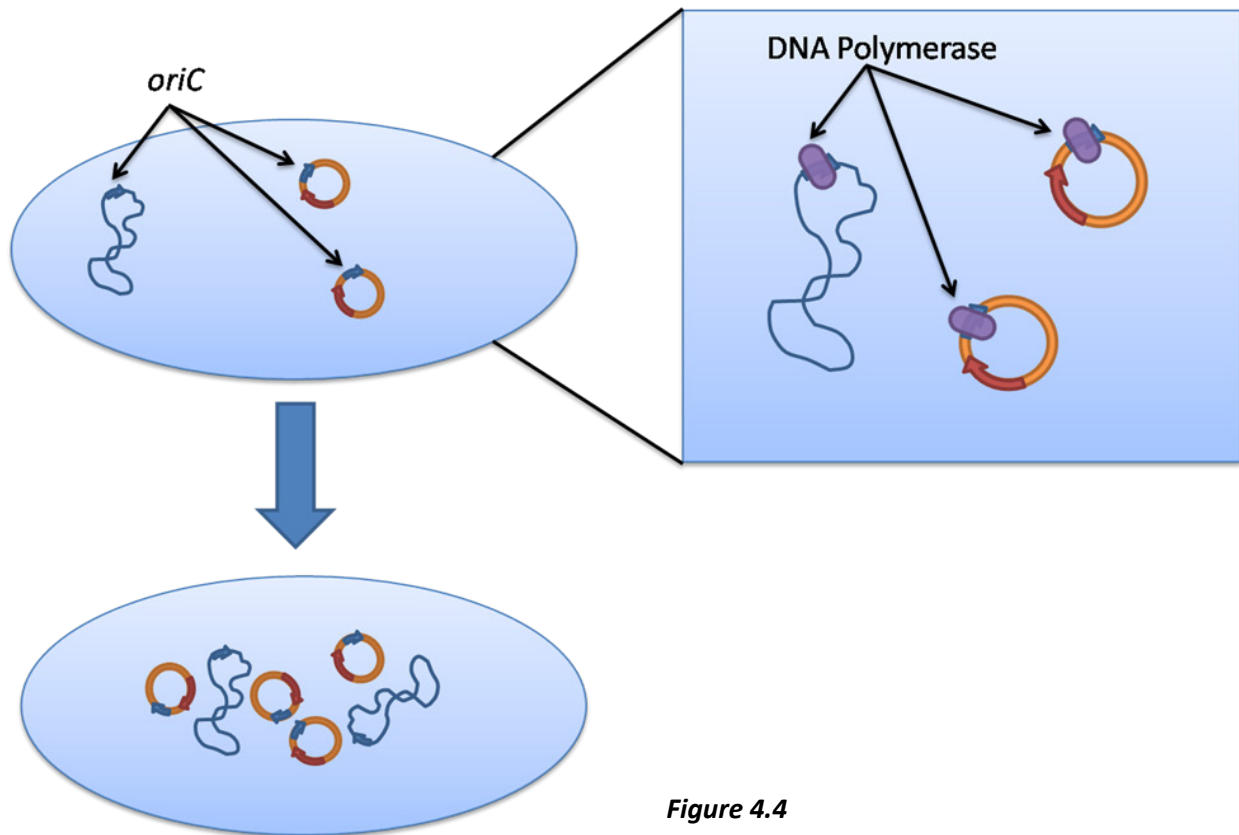

Figure 4.3

Figure 4.4

and prepare the cells for shock by cooling the mixture to 4˚ Celsius, the temperature of a mixture of ice and water, which is also the approximate temperature of a refrigerator. Cooling the cells to this temperature makes the rapid change to 42˚ C all the more drastic and "shocking." After incubation on ice, the cells are moved directly to the water bath at 42˚ C. The cells are heated up rapidly in the water bath, and in their stressed state, resort to taking up DNA from outside the cells in hopes that it will help them survive.

   After the heat shock, some of the bacteria will have taken up the ligated plasmid. Remember that the ligated plasmid contains a gene encoding a protein that gives the bacteria resistance to the **antibiotic** ampicillin, which we will discuss more about in the next chapter. For the bacteria that have taken up the plasmid, it will take some time for the bacteria to generate enough of this protein to protect it from the antibiotic. Therefore, we let the bacteria **recover** from their shocked state by moving them to a non-stressful broth called SOCS at their optimal growing temperature, which is 37˚ Celsius, the temperature of the human body. After recovery, plating cells on an ampicillin-containing **agar plate** will kill off any of the bacteria that had not taken up the plasmid encoding the antibiotic resistance gene. To spread bacteria onto the plate, simply place some bacteria in SOCS onto the plate, and use the **spreader** to gently distribute the liquid around the plate until most of the liquid is absorbed into the agar (Chapter 4 video).

Pre-Lab Questions

1. Transformation of an organism requires the introduction of new genetic material into all of its cells. Therefore, which type of organism would be easiest to transform: bacteria, plants, mice, or humans?
2. Thinking about bacterial cell and human cell responses to their environments, you perform an experiment that highly stresses both types of cells (such as heat shock, electoporation, or

repeated freezing and thawing) and find that bacteria will take in new DNA, but human cells are much more likely to die in response to these stresses. Why do you think this difference could occur, based on what you know about multi-cellular and single-celled organisms?

Lab 4: Transformation

Purpose
Today you will perform heat shock transformation on chemically competent *E. coli* bacteria. Using a beaker of warm water you will force the bacteria to take in your ligated GFP-containing vector from the last lab.

Materials
Equipment
- 2-20µL Air displacement pipette
- Pipette tips
- 1 transfer pipette
- 1 hot plate, beaker with water, and thermometer OR 1 water bath
- 1 tube float (optional)
- Ice bucket (One per lab table recommended)
- 1 microcentrifuge tube per student

  Equipment for Tomorrow
- 1 agar plate containing ampicillin and arabinose
- 1 bacteria spreader

Reagents
- Ligation Reaction from last session
- *E. coli* bacteria on ice
- Tube of SOCS broth

Methods
The laboratory today will be the most time-consuming of all the lab sessions, so in order to finish on time you must be organized, quick, and careful. You will be using your ligation reaction from last week to transform *E. coli* bacteria, recover those bacteria, and then plate them on an agar plate containing arabinose.

When handling live *E. coli* bacteria, be sure not to touch the bacteria with any part of your body, and quickly dispose of any pipette tip, spreader, or transfer pipette that touches the bacteria in a trash can lined with a plastic bag. These *E. coli* bacteria do not cause disease, but you still should not get them on yourself. If your classroom has them, wear gloves and goggles when handling bacteria.

1. ***E. coli* bacteria should remain cold at all times, your instructor will remove the bacteria from the freezer and thaw them on ice.**
2. Use your Air Displacement Pipette to **add 40.0µL of bacteria** from the lab stock tube to your microcentrifuge tube**.**
3. Let your bacteria sit on ice for 2-3 minutes to make sure they stay cold.
4. **Thaw your ligation reaction** from the last lab.
5. Use a pipette to **add 10.0µL of the ligation reaction to the bacteria**.
6. Cap and flick the tube a couple times to mix and then put the mixture with bacteria on ice.

7. Leave the tube on **ice for 20 minutes**, optimally the tube would be left on ice for 1 hour. During this time **re-watch the Chapter 4 Video**.
8. While the bacteria DNA mixture is on ice, heat a beaker of water to 42˚C or prepare your water bath
9. Incubate your bacteria for 60 seconds in the 42˚ water. Don't let the bacteria spend much time at room temperature. Move them straight from the ice bucket into the water bath.
10. Remove the now-transformed bacteria from the water bath. Use the transfer pipette to add around 500µL of SOCS broth to the transformed bacteria. Place the bulb pipette on your bench to use again in ten minutes. Be sure not to mix your bulb pipette up with anyone else's pipette.
11. Tightly cap the microcentrifuge tube containing your transformed bacteria.
12. Holding the transformed bacteria in your hand to warm them to 37˚C (body temperature) and shake the bacteria in an orbital motion for about a minute, then place the tube in the incubator at 37˚C. You should come back **tomorrow morning** (at least 2 hours later) to plate the cells on the agar plate.
13. Write your name on the bottom of your agar plate in permanent marker.
14. Use the bulb pipette to add all of the bacteria in SOCS broth to the center of your agar plate and use the spreader to evenly distribute the liquid around the agar plate. Place the lid back on the plate.
15. **Let the plate sit** for about a minute to absorb some of the liquid, then place it face up in the 37˚C incubator.
16. Tomorrow, after colonies have grown, the plates should to be moved from 37˚C to the refrigerator to prevent overgrowth of bacteria. If you don't see any colonies, try leaving the plates in the incubator for another day (also double check that the incubator is at the appropriate temperature).

Clean-up
Make sure that all of the tubes containing *E. coli* bacteria are properly disposed of and that the ligation reaction is discarded. The microcentrifuge tube that contained your *E. coli* should be tightly capped before being thrown away. Be sure to put away the hot plate and beaker in addition to your pipette, as normal. Make sure the transfer pipette and spreader that you used are thrown away as well. Double check that your initials are on your agar plate and place it in the incubator.

Hand in your group's air displacement pipette to your instructor; you will not need it for the rest of the module.

Conclusions
Today you have inserted some circular plasmid DNA from a ligation reaction into *E. coli* bacteria. By plating the bacteria on ampicillin containing plates, only the bacteria that have the ampicillin resistance gene from the plasmid DNA will be able to grow. By tomorrow you should be able to see individual colonies started by a single bacterium on the plate, and hopefully some of them will express GFP!
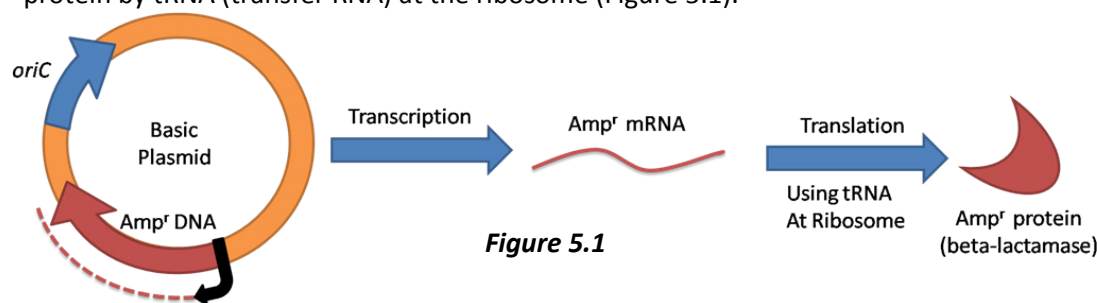
Chapter 5: Selection and Expression

Introduction

You have now amplified the GFP gene, ligated the gene into a plasmid, and transformed that plasmid into bacteria. In this chapter we will cover the details of how the antibiotic selection process works. We will also explore the mechanism by which the GFP protein is produced in the bacterial cells, including genetic regulatory elements controlling gene expression.

Today in lab you will visualize the glowing protein you have made in bacteria, select one colony expressing GFP, and use the bacteria in that colony to draw a fluorescent picture with glowing cells!

Background and Key Words

To this point in our experiments we have only been concerned with DNA, the genetic blueprint that is passed on from one organism to the next. However, if DNA is the blueprint of an organism, then messenger RNAs are the builders, who read the blueprints, and proteins are the building blocks of an organism. Proteins perform almost all of the active functions in a cell, from making and repairing DNA to serving as channels for ions to pass through. DNA genes are **transcribed** into mRNA (messenger RNA), and the mRNA is then **translated** into protein by tRNA (transfer RNA) at the ribosome (Figure 5.1).

Figure 5.1

Key Words
Transcription
Translation
Expression
Ampicillin
Amp^r
Promoter
Arabinose
Operon
Colony
Clone

Every cell of a multi-cellular organism has nearly the same DNA, but different cells, like a neuron and a skin cell, express vastly different mRNAs and proteins. When someone talks about the **expression** of a gene, they are referring to the amount of mRNA being made from that gene.

We mentioned in the last chapter that the plasmid construct that was transformed into bacteria contained a gene that gives bacteria resistance to the antibiotic **Ampicillin**. This resistance is conferred by the gene **Amp^r** in the plasmid DNA (for "Ampicillin Resistance"), which encodes the mRNA that is then turned into the protein beta-lactamase, which blocks the function of ampicillin (Figure 5.2). In order for the cell to be protected from ampicillin, the Amp^r gene must be expressed rather than simply present. In other words, because Amp^r is expressed in bacteria that have the plasmid, those cells are protected from ampicillin. If Amp^r were not expressed, the bacteria would die, even if the cell had the DNA encoding the Amp^r gene.

Our plasmid is built to express the Amp^r gene constitutively, which means that mRNA and protein for Amp^r is being produced all the time in the cell. However, the regulation of expression of GFP in our plasmid is slightly more complex. Gene expression is controlled by a **promoter**, a nucleotide sequence in

Figure 5.2

+ Ampicillin

+ Ampicillin

Ampicillin kills bacterium

Beta-lactamase protein from Amp^r protects bacterium
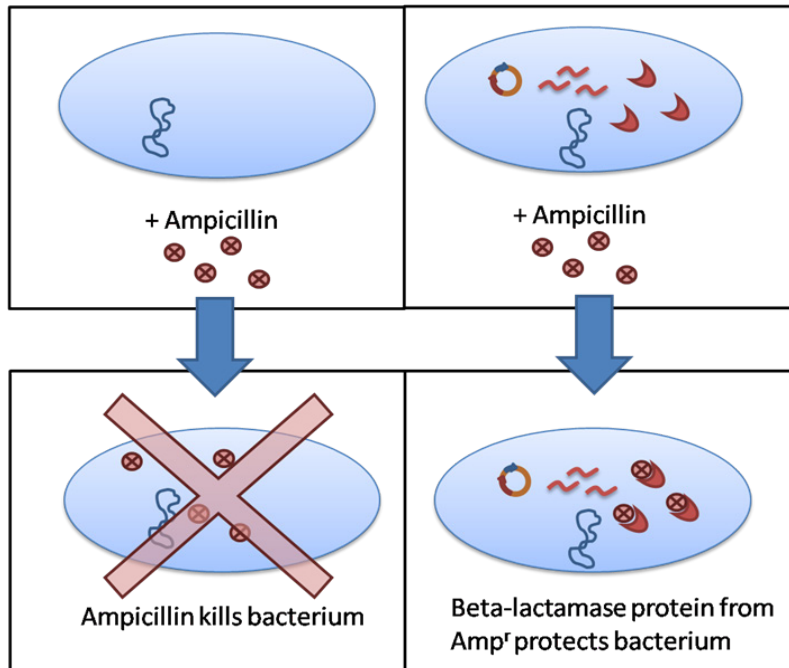
the DNA that signals when a specific gene should be expressed. Our bodies do not want all our proteins expressed continuously. Expressing eye proteins in the gut or insulin in the skin is both a waste of energy in the body and could have possible detrimental effects. Therefore, genes have evolved to be controlled by promoters, which only express the gene when it should be expressed. Promoters are generally activated when a protein recognizes the DNA sequence of the promoter and calls in the enzyme **RNA polymerase** to begin the transcription of the genes into mRNA (Figure 5.3).

Although single-celled organisms like bacteria do not have different tissues in which they will need to express different genes, bacteria also possess promoter sequences to control the expression of genes, like mammals. Most of these promoters in single-celled organisms are turned 'on' or 'off' by the presence of a molecule normally external to the cell. For example, the sugar **arabinose** can be used as a food source for *E. coli* bacteria, but in order to process arabinose as a food source, three genes need to be expressed: *araA, araB,* and *araD*. To express these genes, the protein made from a fourth gene, *araC*, must interact with unbound arabinose. *araC*, which is expressed constantly, can bind to the promoter sequence of the other three genes only when it first binds arabinose. After binding to arabinose, *araC* protein undergoes a conformational change which allows it to bind to the promoter site for the other *ara* genes and recruit RNA polymerase to begin their expression (Figure 5.4). These three genes working in concert are known as an **operon**. Arabinose, in this system, is called an **inducer**, because its presence induces the expression of the arabinose operon. By using this system, the bacteria can have 3 genes "in storage," ready to use at a moment's notice, while only spending the energy to make one protein all the time!

In our plasmid construct, we make use of this property of the *araC* gene to explore the mechanisms of gene regulation. Our GFP gene that we have cloned into the plasmid should now be under the control of the arabinose operon promoter sequence. Therefore, GFP will not be expressed unless *araC* bound to arabinose sugar recruits RNA polymerase to begin transcription. To that end, our plasmid already contains the *araC* gene under a constitutive promoter, to make sure there is plenty of it in our *E. coli* cells. Therefore, if the bacteria are grown in an environment that contains arabinose, and if the GFP plasmid is present, GFP mRNA should be made. However, if there is no arabinose in the
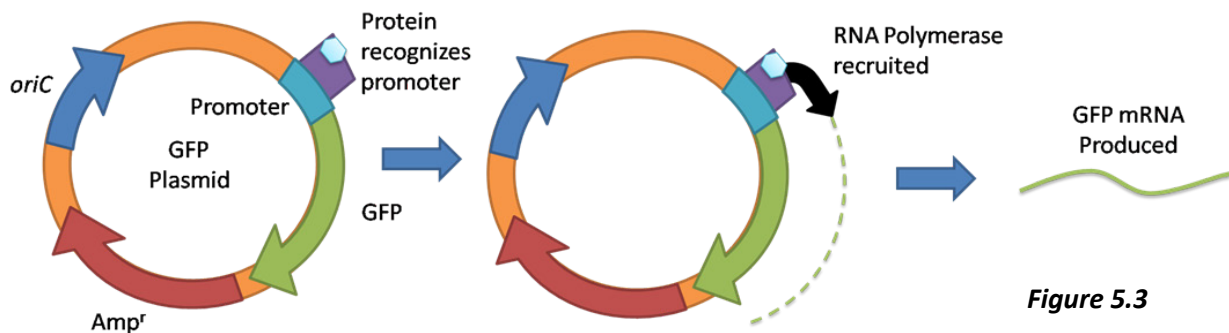

oriC

Protein recognizes promoter

Promoter

GFP Plasmid

GFP

Amp^r

RNA Polymerase recruited
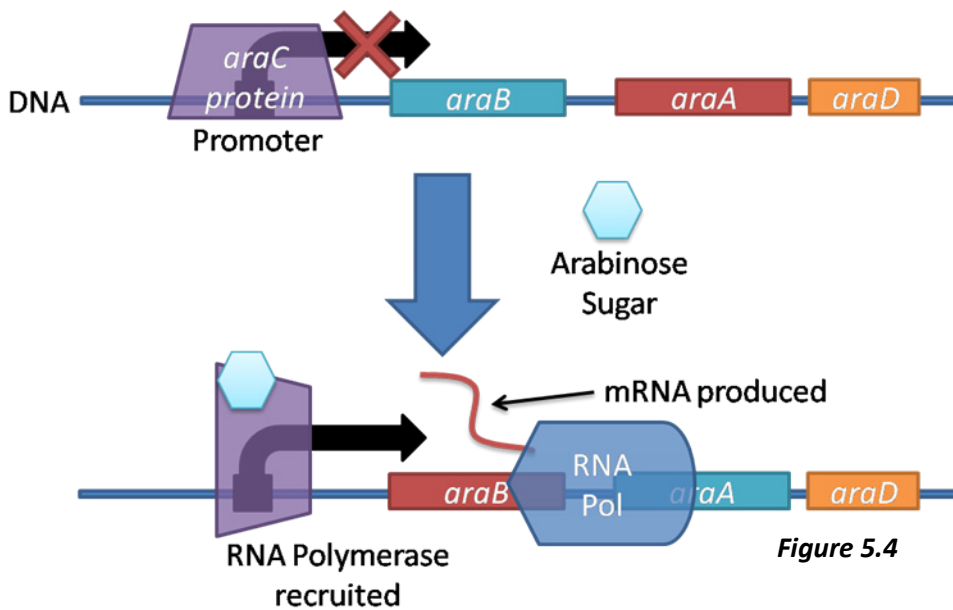
GFP mRNA Produced

Figure 5.3

Figure 5.4

bacterial environment, there will be no GFP expression (Figure 5.5).

Now that we know more about how a promoter drives the expression of GFP mRNA in our plasmid, let's think about the possible results from the transformation lab that you have just finished. Looking back at the experimenting you have done so far, the ligation mixture contained the GFP PCR insert and the linearized vector, exposed to the enzyme DNA ligase. Let's look carefully at what different types of vector and insert combinations could go into bacteria after ligation, and what the outcomes would be (Table 5.1).

On the agar plate with ampicillin, a single bacterium which is transformed by a plasmid giving it resistance to ampicillin will replicate about every thirty minutes at 37 degrees Celsius. Since its daughter cells will each divide again every thirty minutes, a single bacterium will establish a **colony** of millions of identical bacteria after a day of growth. Because each of these bacteria in the colony is exactly like the founder bacterium, this colony is called one **clone**. Because we will select one clone from the agar plate which has our desired insert, the entire process that you have performed is called cloning.

Although the linearized vector DNA or the linear PCR product could be taken up by the bacterial cell instead of the desired plasmid, only bacteria that have taken up the circular plasmid will be able to replicate the plasmid, survive, and form colonies. However, because your GFP PCR product has the same overhanging Adenosines at both ends, the insert can ligate into the vector backwards. If that happens, the *araC* promoter will try to make mRNA from a backwards GFP DNA sequence, which cannot be translated into GFP protein. This means that about ½ of your colonies will not express GFP, since the construct can go in backwards! We will be detecting the orientation of GFP in our plasmid by looking for the GFP protein using UV light: only clones with GFP in the correct orientation will make GFP protein.
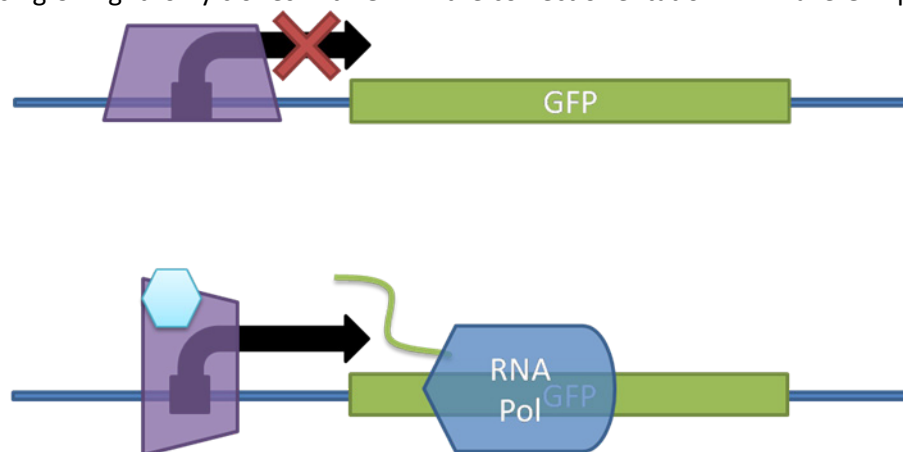

Figure 5.5

Table 5.1

Combination	Outcome
GFP inserts into vector	Bacteria survive, form colonies, and express GFP
GFP inserts into vector backwards	Bacteria survive and form colonies
Linearized Vector	Some bacteria survive, but can not divide because plasmid cannot replicate
Linearized GFP	Bacteria do not survive
No transformation	Bacteria do not survive

Pre-Lab Questions

1. Design two simple experiments using LB agar plates, arabinose, ampicillin, and a GFP-vector ligation similar to the one you performed in this lab to demonstrate that *E. coli* are efficiently killed by ampicillin and only express GFP in the presence of arabinose. Make sure these experiments have appropriate controls.

2. Only a portion of the DNA from the vector and the PCR is correctly ligated and allows the bacterium which takes up the DNA to survive and proliferate to form a clone. Imagine for a moment that instead of using sticky end cloning described in the last chapter, you were instead using Blunt End cloning, where any end of DNA could stick to any other end in the solution. What types of plasmids could be observed in the ligation reaction you have performed? For example, one option is a plasmid made up of vector and insert GFP in the correct orientation, which looks like this.
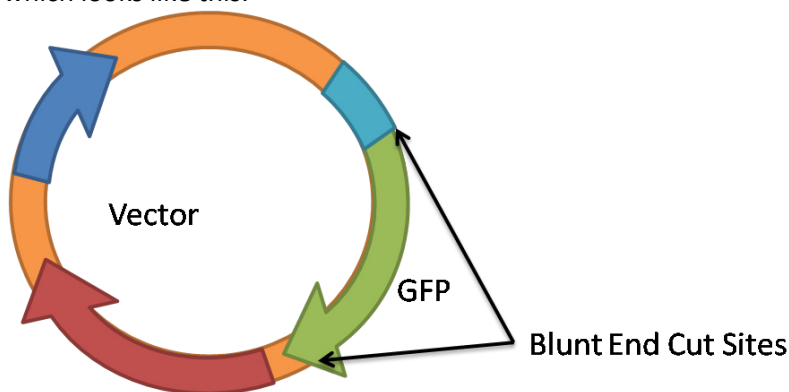
   Draw at least four other possible plasmids that could be created by this blunt-end ligation reaction. Use Table 5.1 as a guide. What options are present here which are not present in table 5.1? Why?

3. Why does the plasmid need to be circular in order for the bacterium that takes up the plasmid to form a colony?

4. (Optional) Today you will be drawing a picture in fluorescent bacteria that you will grow overnight. If you want, you may draw the design before class to use as a template when you paint the design with bacteria. Turn to chapter 6 to see some examples of designs by previous students (Figure 6.3).

Lab 5: Selection and Expression

Purpose
Today you will be visualizing the expression of Green Fluorescent Protein in bacterial colonies using a long-wave UV light. You will select one colony that expresses GFP (a successful transformant) and use bacteria from that colony to draw an elaborate picture of your own design.

Materials
- Agar plate with GFP expressing *E. coli* colonies from last lab.
- Long-wave UV lamp
- 1 Agar plate with ampicillin and arabinose
- 1 mL tube of LB broth
- Agar plate painting tools, which include but are not limited to
  - Pipette tip
  - Toothpick
  - Cotton swab

Methods
In today's session you will visualize the expression of GFP in your bacterial colonies using a UV light and then use a GFP-expressing clone to paint an entire picture in fluorescent bacteria which will grow up within a day.

When handling live *E. coli* bacteria, be sure not to touch the bacteria with any part of your body, and quickly dispose of any pipette tip, toothpick, or cotton swab that touches the bacteria in a trash can lined with a plastic bag. These *E. coli* bacteria do not cause disease, but you still should not get them on yourself. If your classroom has them, wear gloves and goggles when handling bacteria.

When using a Long-wave UV lamp, always keep the light pointed away from yourself and other people. If your classroom has them available, use goggles when handling the UV light. Long-wave UV light is less damaging to cells than the higher energy short-wave UV light, but exposure should be minimized nonetheless.

Before painting your fluorescent picture, you may want to check out Figure 6.3 in the next chapter, to see what other students have drawn with this same kit!

1. Retrieve your agar plate from last lab session; it should have a number of small round, whitish colonies growing on it.
2. Shine the long-wave UV lamp on the plate, and observe to see if you have any GFP-producing colonies, they should glow green when illuminated by the long-wave UV light.
3. On the bottom of the plate, use a permanent marker to circle one of the colonies that is expressing GFP. You will use this clone to paint your picture in bacteria.
4. Open the lid of your plate, and using a toothpick or pipette tip, gently touch the colony you are going to use, then mix the bacteria on the end of your tip or toothpick into the microcentrifuge tube of LB Broth.
5. Discard that tip or toothpick, close the lid on your tube of LB with bacteria in it, and replace the lid of your agar plate from last week.
6. Invert the tube containing LB and bacteria multiple times to ensure that they are well mixed.

7. Open the tube with LB and bacteria. Then touch the painting tool you are going to use to the LB in the tube. The microscopic bacteria in the LB will be lifted out on the end of the pipette tip, toothpick, cotton swab, or other instrument, allowing you to paint a detailed and beautiful picture onto the new agar plate!
8. Optional: if you have a detailed idea of the picture that you want to create, it may help to draw it out on paper first, and then place the paper underneath your agar plate while you are adding the bacteria in whatever design you choose.
9. When you are finished painting, be sure to correctly dispose of all of your painting tools and your bacteria in LB broth. Then invert your agar plate and place it in the 37˚ Celsius incubator.
10. The cells should be allowed to grow for anywhere between 12 and 24 hours, if the plates need to be kept for longer than this, they should be moved to the refrigerator after the 12 to 24 hours. An agar plate sealed with plastic wrap can last in the refrigerator for over a month.

Clean-up

Make sure that all agar plate painting tools are properly disposed of in the trash can. The microcentrifuge tube containing LB and the bacteria should be tightly capped and thrown away. Make sure that your agar plate has your initials before placing it in the incubator to grow.

Conclusions

Congratulations! You have successfully cloned the GFP gene from a template into a vector and then grown in bacteria. This technique is used by thousands of scientists to manipulate DNA for a broad range of experiments, and now you are among their ranks. If everything has worked perfectly to this point, you have done exceptionally well and worked carefully and accurately. Enjoy your fluorescent picture made of living organisms that will be ready to look at tomorrow.

You are also now ready to take the final assessment for the course, which will involve the application of many of the concepts you have learned during the course. Your instructor may distribute the final assessment today before you leave the laboratory.

Chapter 6: Fluorescence

Introduction

You have now successfully cloned the GFP gene into a bacterial expression vector, and you have used genetic regulatory elements to control the expression of that gene. In the last laboratory session you should have been able to visualize the GFP protein being expressed in bacteria by exciting the protein using a UV light.

In this, the final chapter of *Cloning a Fluorescent Gene*, you will learn how fluorescence works, what different types of technologies are available which use fluorescence, and some ways that fluorescence is being used in modern scientific laboratories.

Background and Key Words

By now you have seen the dramatic fluorescence of the GFP protein expressed in bacterial cells. You also know that UV light is required to elicit GFP's green color. Ultraviolet light and green light are both parts of the **electromagnetic spectrum**, which includes all types of radiation frequencies. You may know from physics that the frequency of electromagnetic radiation inversely correlates with its **wavelength**, which is the distance from one peak of the wave to the next. Shorter wavelengths give more intense radiation and longer waves have less energy. For example, x-rays, which have a very short wavelength, carry a large amount of energy and can be harmful if people are exposed to them, but infrared waves do not pose as much of a danger, because the wavelength is longer than that of visible light.

When looking at fluorescent molecules (including proteins like GFP), it is typical to label each of them with two important pieces of information: excitation and emission points. The **excitation** point is the wavelength of light whose energy is absorbed by the fluorescent molecule: long wave UV light at about 380nm for GFP. The **emission** point is the wavelength of light given off by the excited fluorescent molecule: green light at around 500nm for GFP (Figure 6.1). Remember that the emission point of a fluorescent molecule will always be of a longer wavelength than the excitation point of that molecule because it is absorbing some of the energy and emitting a wave with lower energy.

Fluorescent molecules work by absorbing some, but not all energy from a specific type of electromagnetic wave, and emitting the rest as energy with a new wavelength. In this way fluorescence is different than simply being colored. For example, if you are wearing a blue shirt, you can perceive that it is blue when the shirt is being hit by white light (which contains all colors). The reason you can see the color of the shirt is that non-blue colors are being absorbed by the fabric and the blue color is being reflected. However, if you were somehow standing in light that contained no blue light, your shirt would appear black because it would not be reflecting any light.

Key Words
Electromagnetic Spectrum
Wavelength
Excitation
Emission
Fusion protein
Transgenic

Wavelength (meters)

10^{-14}	Gamma rays
10^{-12}	X-rays
10^{-10}	
10^{-8}	Ultra-Violet
10^{-6}	
	Infrared
10^{-4}	
10^{-2}	Micro-waves
1	Radio Waves
10^{2}	

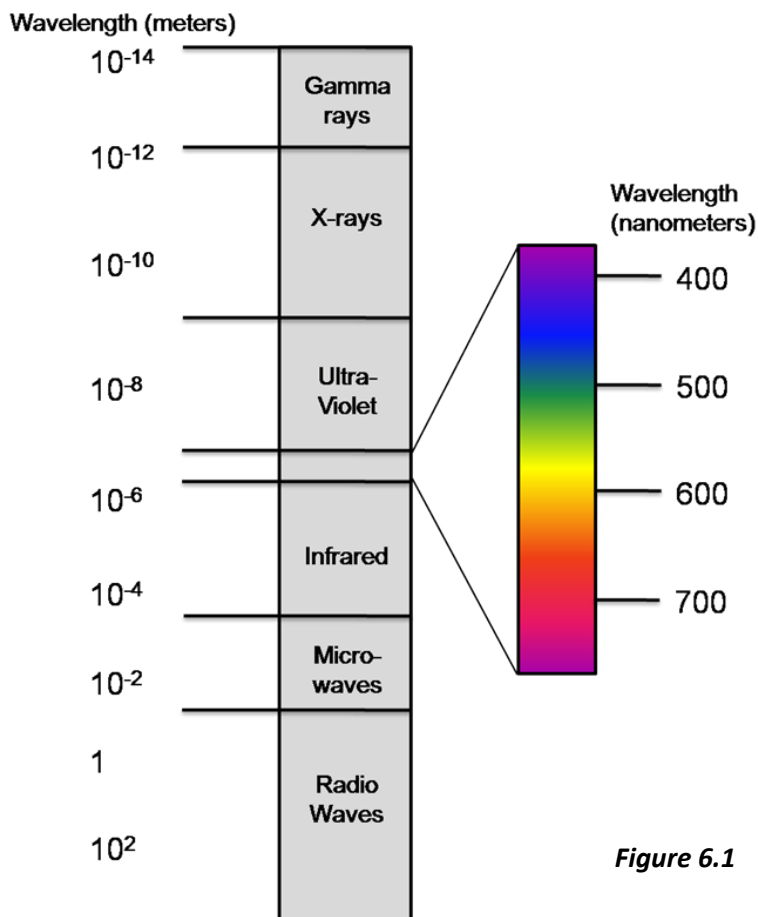**Wavelength (nanometers)**

- 400
- 500
- 600
- 700

Figure 6.1

Fluorescent molecules work in a fundamentally different way, because they can still be seen even when there is no light of the color they are emitting. For example, GFP is excited by UV light and emits green light, so even without any green light being provided, UV light will make GFP glow green (Figure 6.2).

Different fluorescent molecules have vastly different excitation and emission points, which allow for a dizzying variety of fluorescent molecules to be used at the same time. Some examples include mutants of GFP, where a few amino acids of the proteins were changed and the emission and excitation colors of the molecule completely changed, such as BFP (Blue Fluorescent Protein), YFP (Yellow), and CFP (Cyan).

The first breakthrough with fluorescent proteins, as opposed to fluorescent dyes, had to do with exactly the sort of system you used in this experiment. Scientists put the gene for a fluorescent protein downstream of a promoter, so that the gene is only expressed when the gene that promoter normally controls would be expressed. Other fluorescent molecules used before GFP were chemicals which could not be manufactured by mammalian cells. However, after GFP was cloned in 1992, researchers for the first time were able to visualize with fluorescence exactly which cells were expressing a specific gene. This tool has been incredibly important in the development of cutting edge science in the last decade, and this work has been commemorated by the 2008 Nobel Prize in Chemistry, which was bestowed upon three researchers instrumental in the development of research tools using GFP.

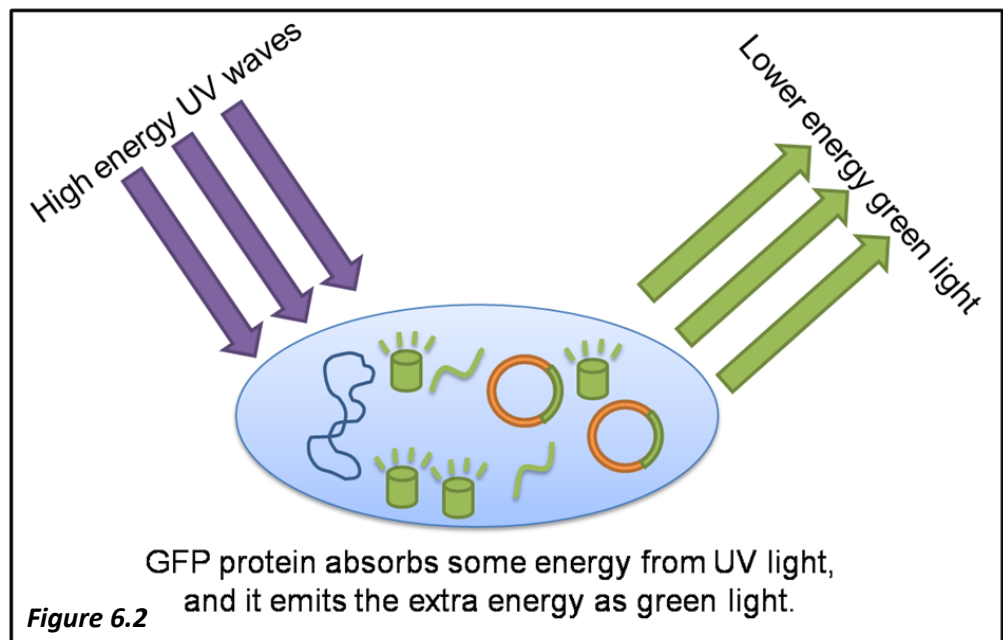Although painting a fluorescent picture using GFP expressing

High energy UV waves

Lower energy green light

GFP protein absorbs some energy from UV light, and it emits the extra energy as green light.

Figure 6.2

bacteria (Figure 6.3) doesn't answer any important scientific questions, this cloning process does demonstrate many of the critical steps in the utilization of fluorescent proteins in scientific laboratories. This exercise is also a fine demonstration of the power of biotechnology to manipulate genes and gene expression, a tool that is revolutionizing medicine and agriculture. If you choose to do biology research in the future, you will almost certainly encounter these two extremely common tools: Cloning and Fluorescence.

Figure 6.3

Pre-Lab Questions

1. If a fluorescent protein has an excitation point at wavelength 500nm, and an emission point that is 80nm from the excitation point, what is the wavelength of the emission point? What color of light does this protein emit?
2. You know that the plasmid that you have used in these experiments controls the expression of GFP using an arabinose-inducible promoter. Where would you look for a promoter if you wanted to create a plasmid that only expressed GFP in human skin cells?

Lab 6: Fluorescence

Purpose
Observe the designs made by you and your classmates using a UV lamp.

Materials
- Long-wave UV lamp
- Camera (optional)

Methods
Today you will simply be observing the fruits of your labors. Collect your agar plate from the incubator and use the UV light to illuminate your design! Compare your design with those of your classmates, and use a digital camera to take pictures.

If you email your pictures to us at data@otyp.es your art may be included in the next edition of the lab manual!

Clean-up
You may now throw away all of your agar plates and any extra tubes that are in your group's plastic bag in the freezer. Be sure to clean up your lab area with an ethanol-containing cleaning solution, like Windex®, which will kill any bacteria that got onto the lab bench.

Conclusions
This is the end of gen.otyp's *Cloning a Fluorescent Gene*. We hope that you have enjoyed the course and have learned some valuable skills to use in the laboratory. To those of you who are soon going to be in college, and may want to work in biology research laboratories, be sure to mention that you have experience doing PCR and cloning when applying for a job as a research assistant, it may help you secure the position! Best of luck to you all.

Glossary of Terms

5x PCR Buffer: A stock solution of salts that allow for the enzymatic activity of the polymerase Taq. This solution is diluted 1:5 to achieve its final concentration.

Agar Plate: A petri dish filled with LB containing a solidifying agent, generating a semi-solid surface on which bacteria can easily grow.

Air Displacement Pipette: A device which uses the creation of a partial vacuum to lift and move various volumes of liquid.

Amp^r: Ampicillin resistance gene, the gene encoding the protein beta-lactamase, which inhibits the death of bacterial cells induced by the antibiotic ampicillin.

Ampicillin: An antibiotic commonly used in the laboratory for the selection of bacteria.

Amplification (in PCR): the process of increasing the total number of copies of a single DNA strand between two primers.

Annealing (in PCR): the process of attaching short DNA primers to ssDNA that have been denatured

Antibiotic: A molecule capable of killing bacteria which are exposed to it.

Arabinose: A sugar molecule capable of binding to the *araC* protein and inducing the arabinose promoter to express genes following it (such as the arabinose operon or GFP).

Blunt End: A DNA fragment ending in dsDNA with no overhanging nucleotides.

Calcium Chloride: A salt solution used to increase the competence of bacterial cells in heat shock.

Clone: A group of bacteria all possessing the exactly same genetic material.

Colony: A large number of bacteria growing on an agar plate which arise from a single founder bacterium.

Competence: The ability of cells to take up DNA from its environment.

Denaturation (in PCR): the process of unzipping dsDNA into two pieces of ssDNA

DNA Directionality: the concept of DNA being replicated and transcribed starting from the 5' phosphate end and adding to the 3' hydroxyl end

DNA Polymerase: the enzyme that is responsible for making new DNA in all living cells.

DNTPs: **D**eoxyribo-**N**ucleotide **T**ri**P**hosphate**s**, the free floating Adenine, Thymine, Guanine, & Cytosine nucleotides.

dsDNA: Double stranded DNA

Electromagnetic Spectrum: The range of all possible electromagnetic radiation wavelengths.

Elongation (in PCR): also called **Extension;** the process of extending the 3' end of the primer with a DNA polymerase by adding new nucleotide bases that complement the bound ssDNA template

strand. This results in the generation of a dsDNA fragment from an original ssDNA fragment bound to a primer.

Emission: The process of a fluorescent molecule giving off light of a different wavelength than it was exposed to.

Excitation: The process of inducing the absorption of light by a fluorescent molecule.

Expression: The generation of mRNA from a gene which can later be made into protein.

Extension: See [Elongation]

Fusion protein: An artificial protein composed of two different proteins or parts of proteins transcribed and translated in a continuous string.

Heat Shock: The process of transferring of bacteria in a salt solution from a cold to hot environment very quickly, resulting in stress and uptake of DNA from the environment.

Insert: The piece of DNA that will be newly inserted into the plasmid

LB: Lysogeny Broth, a bacterial growth medium

Ligation: the joining of two fragments of dsDNA by the formation of new phosphodiesterbonds.

Microcentrifuge Tube: A plastic capped tube which can hold up to 1.5 mL of liquid (1,500μL) and are named because they are designed to fit into microcentrifuges, which can spin them around fourteen thousand times per minute.

Microliter: A small measurement of volume equivalent to $1/1000^{th}$ of a milliliter

Operon: A collection of genes controlled by a single promoter.

Origin of Replication: DNA sequence that starts the replication of the bacterial chromosome or plasmid.

PCR Tube: A tube that holds 0.2 milliliters (200μL) or 0.5 milliliters (500μL), and is perfectly sized to fit into the blocks of thermal cyclers used in PCR

PCR: Polymerase Chain Reaction, a process which amplifies a short sequence of DNA between two known primers by using thermal cycling to denature, anneal, and extend the DNA fragments.

Plasmid: Small circular piece of DNA containing genetic material which can be replicated in bacteria.

Primer: A short DNA oligonucleotide that binds to denatured DNA at a specific site, providing a "start" signal for a polymerase.

Promoter: The region of DNA responsible for controlling the expression of the gene that follows it.

Recover: The process of allowing bacteria to recover from the stress induced by electroporation or heat shock by shaking them in LB with no antibiotic.

Restriction Enzyme: a protein which recognizes a specific base pair sequence of DNA and cuts dsDNA at those conserved points

Spreader: A tool used for distributing bacteria around an agar plate evenly.

ssDNA: Single stranded DNA

Sticky End: A DNA fragment ending in either 5' or 3' overhanging ssDNA nucleotides.

Taq: A thermostable DNA Polymerase isolated from the Archaebacteria *Thermus aquaticus*, an organism found in the "Great Fountain" of Yellowstone National Park.

Transfer Pipette: A plastic tube with a soft bulb that is squeezed to expel air, inserted into liquid, and released to draw that liquid into the tube and bulb of the pipette.

Transformation: The process used to alter an organism's phenotype by introducing new genetic material.

Transcription: The process by which DNA is read by the RNA Polymerase enzyme to produce messenger RNA. This process can also be referred to as 'expression' of a gene.

Transgenic: The insertion of a new promoter-gene combination into the chromosomes of an organism.

Translation: The process by which messenger RNA is read at the ribosome by transfer RNA bound to amino acid residues, which links together amino acids to produce proteins.

Vector: The backbone of a plasmid, containing all of the pieces it needs to work but without any experimental gene inserted into it.

Wavelength: the distance between repeating units of a wave of a given frequency.

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